

# The role of hypoxia in regulating the formation and activity of bone cells

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## *Abstract*

Hypoxia occurs when the blood supply to tissues is reduced or disrupted. In normal tissues  $pO_2$  is in the range 5 – 12%; however in diseased tissue this value can fall to below 1%  $O_2$ . Much pathology associated with bone loss is also associated with decreased local or systemic  $pO_2$ , such as infection, inflammation, and cancer. The aim of this thesis was to determine the effects of decreased  $pO_2$  on the formation and activity of osteoclasts (OC), the bone resorbing cells, and osteoblasts (OB), the bone forming cells. Exposure of murine bone marrow monocytes, cultured in the presence of M-CSF and RANKL, to  $pO_2$  in the range 20% to 0.2% led to dramatic increases in osteoclast formation at 1 - 2%  $O_2$ , with significant increases even at  $pO_2$  as low as 0.2% (severe hypoxia), over those cultured at 20%  $O_2$  (normoxia). Decreased  $O_2$  did not activate mature OC isolated from neonatal rat bones to resorb bone, indicating that hypoxia has no effect on osteoclast activity. When the effects of hypoxia were tested on human OC formation from peripheral blood monocytes, a similar trend was found to that observed in rodents, indicating that hypoxia is acting directly on OC rather than stromal cells present in the marrow cultures, in support of this result, the effects of hypoxia were dependent on permissive levels of M-CSF and RANKL. In contrast to its stimulatory effects on OC, hypoxia inhibited the production of mineralised nodules in primary rat OB cultures in a dose dependent manner, with almost complete abolition of nodule formation at 0.2%  $O_2$ . Proliferation of rat OB, measured by tritiated thymidine incorporation at days 6, 12 and 18 of culture, was inhibited by 50 - 70% in 2%  $O_2$  as opposed to culture at 20%  $O_2$ ; however, apoptosis was not altered by culturing OB at 2%  $O_2$ . Transmission electron microscopy of nodules revealed dense, regular fibrils of type I collagen in 20%  $O_2$  cultures, whereas this ultrastructure was disorganised in collagen formed in hypoxia. Collagen production was decreased and reduced lysine hydroxylation within the deposited collagen was observed when OB were cultured at 2%  $O_2$ . Furthermore OB expressed lower levels of the lysyl hydroxylase enzymes in cultures maintained at 2%  $O_2$ . The percentage of proline hydroxylation in deposited collagen was unaffected by decreased  $O_2$  availability. Expression of collagen type 1 $\alpha$ 1 was unaffected by hypoxia, as was expression of all transforming growth factor  $\beta$  isoforms; however expression of the differentiation markers osteocalcin and alkaline phosphatase were delayed in hypoxic cultures, and OB alkaline phosphatase activity failed to increase with time in hypoxic cultures, suggesting that differentiation of OB is delayed by hypoxia. Together these results indicate that hypoxia induces in OB a state of 'suspended animation' where cellular energy is directed towards survival rather than bone formation. The reciprocal nature of the responses of OC and OB to hypoxia would lead to marked bone loss in situations where oxygen tension is reduced and may explain the bone loss associated with a variety of pathological situations. These results emphasise the importance of the vasculature in maintaining bone health.

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# CHAPTER 1

## *General Introduction*

Oxygen is essential for the processes that sustain cell activity and viability. It acts as an electron acceptor during mitochondrial respiration, and as such is necessary for energy production; it is also a substrate in a variety of enzymatic reactions. The method by which oxygen is supplied to cells is through the vasculature. It has been well established that bone formation, developmentally and during maintenance and repair, requires a functional vascular network; however, the exact role of this vascular network and its influence on bone formation and destruction are not well understood. Oxygen tension in bone marrow aspirates has been measured at 55 mmHg (~7% O<sub>2</sub>) (Harrison *et al.*, 2002; Ishikawa and Ito, 1988) in healthy volunteers. Many conditions that are associated with increased bone loss are also associated with decreased pO<sub>2</sub> (hypoxia) such as in the pannus of joints afflicted with rheumatoid arthritis, and at the site of fractures, tumours and infections. Investigating the interaction between bone and its attendant blood supply requires knowledge of the respective roles these systems play within the body and mechanisms by which they are regulated.

### **The role of bone in the body**

The skeleton serves a structural role within the body, providing protection for internal organs and the scaffold upon which the body is supported. The bone matrix and marrow space also serve secondary functions in maintaining calcium homeostasis and acid-base balance, and as the site of haematopoiesis, respectively. Throughout the lifetime of an organism, the bone matrix is in a state of constant turnover, with packets being removed and replaced to maintain its structural integrity and to release bone mineral. These functions require a satisfactory vascular supply to provide

essential nutrients to bone cells and allow egress of calcium and newly formed blood cells into the circulation. The structure of bone is integral to its mechanical and physiological roles.

### ***Molecular composition of bone***

The molecular makeup of bone matrix includes a collagenous organic component and a mineral (hydroxyapatite) component. Type I collagen forms approximately 90% of the organic matrix; the remaining 10% is comprised of a variety of molecules, including structural proteins, for example, osteopontin (OPN), bone sialoprotein (BSP), and osteocalcin (OCN), and growth factors such as transforming growth factor  $\beta$  (TGF $\beta$ ). These molecules serve a variety of roles, including mediating cell - matrix interactions and matrix deposition, and can be released from the matrix during bone resorption and activated to perform signalling roles within the bone microenvironment or further-a-field within the body. The mineral component consists of calcium hydroxyapatite  $\{Ca_{10}PO_4(OH)_2\}$ ; formation of both the organic and inorganic components of bone matrix are regulated by osteoblasts, through their production of extracellular matrix components and enzymes that mediate mineral deposition.

### ***Gross anatomy***

The extremity of the long axis of tubular bones is termed the epiphysis; this adjoins to the metaphysis, which is a boundary region between the epiphysis and the shaft of the bone, the diaphysis. During development, the metaphysis is separated from the epiphysis by the cartilaginous growth plate. A permanent layer of articular cartilage covers the epiphysis. At the macroscopic level, bone consists of several distinct regions. The outer layer of solid bone matrix is termed cortical bone, within the external layer of cortical bone is a meshwork of bony plates termed trabecular or cancellous bone, which extends into, and is surrounded by, the bone marrow.

### ***Bone marrow***

The bone marrow is a site of haematopoiesis, and is the main source of blood cells in healthy adults. The immune functions of the bone marrow govern its



structure, and the mesenchymal cells in the marrow provide a suitable environment for haematopoiesis. Haematopoiesis occurs around sinuses in the vasculature. These sinuses are external to the vessel wall and consist of fat and reticular cells that provide the support structure for the immature cells. As cells progress further down their differentiation pathway they move from the exterior of the marrow shaft towards the central region, they slowly lose their adherent cell surface molecules and are pushed into the circulation through the cytoplasm of endothelial cells lining the sinus (Tavassoli and Yoffey, 1983).

### ***Bone development***

Bone forms from mesenchymal condensates in the developing embryo. These cells proliferate and differentiate under the influence of a variety of growth and differentiation factors, including bone morphogenic proteins (BMPs). The skeletal template or body plan is defined by the temporal and spatial expression of DNA binding homeobox (HOX) transcription factors such as *Msx-1* and *-2*, *Dlx-5* and *-6*, *Hoxa-2*, *Hoxd-9* and *rHox* (de la Fuente and Helms, 2005; Hassan *et al.*, 2004). These regulate the location of bone formation, are expressed during embryogenesis, and reactivated during fracture healing (Gersch *et al.*, 2005). At sites targeted for bone formation by HOX gene expression mesenchymal cell condensates form, at which point bone formation can proceed through 2 separate mechanisms. The first, intramembranous ossification, requires no cartilage intermediate: mesenchymal cells within condensates differentiate directly into osteoblasts. The second, endochondral ossification, involves the production of a cartilage template and the coordinated vascular invasion and mineralisation of that template. Endochondral ossification forms the long bones and is the most prevalent mechanism, whereas the flat bones, such as those comprising the cranium, form via intramembranous ossification.

### **Cells of bone**

There are four major cell types that control the formation and removal of bone; they are the chondrocyte, the cartilage forming cell; the osteoblast, the bone-forming cell; the osteoclast, the bone-removing cell; and the osteocyte, which resides within the bone matrix. The differentiation and activity of these cells are controlled by a

diverse range of signals, which act in concert to balance the multiple functions of bone.

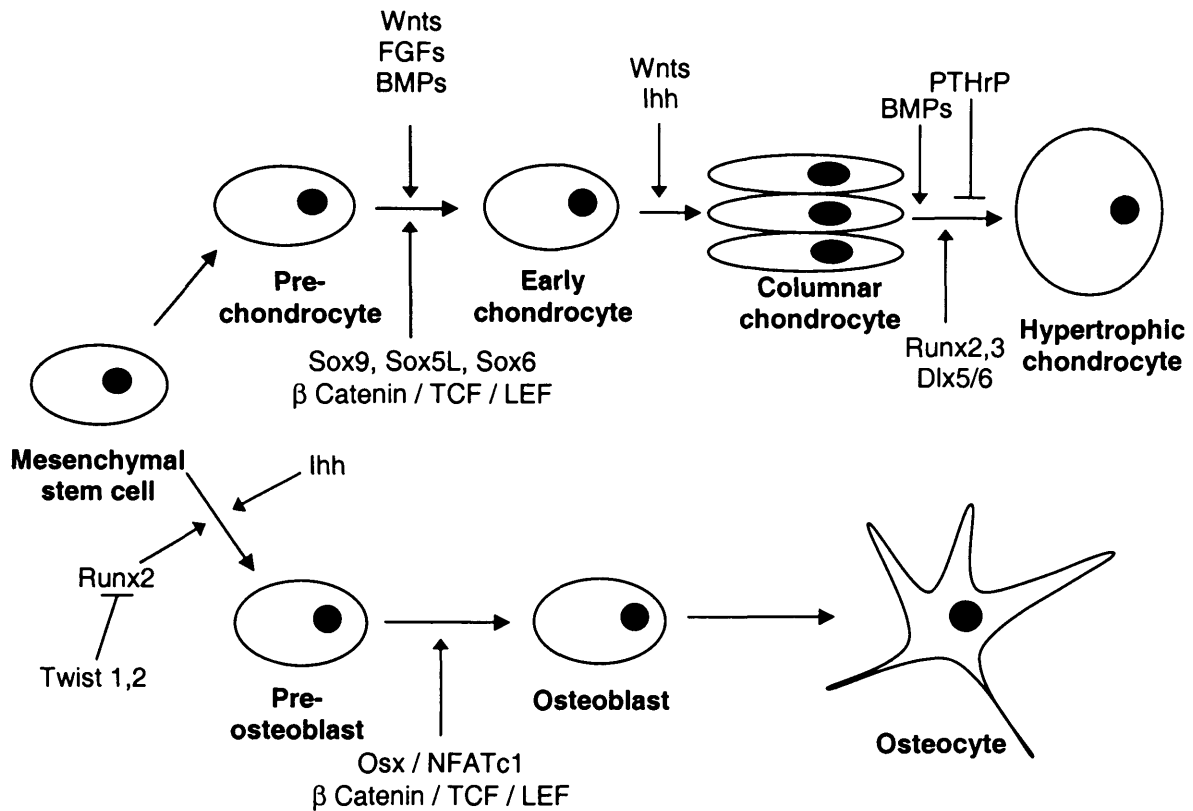
## ***Chondrocytes***

### ***Differentiation***

Cartilage production is required for long bone formation and endochondral ossification; this cartilage is termed 'replacement cartilage', as it is targeted for vascular invasion and, ultimately, bone formation. A second form of cartilage exists, termed persistent cartilage, which is present continuously, and is avascular. Differentiation into the chondrocyte phenotype is controlled by several essential transcription factors (**Figure 1.1**). The determining factor for persistent cartilage formation is Sox9 expression (Eames *et al.*, 2004). Sox5 and Sox6 are also required for cartilage development; although single knockouts show only minor cartilage dysfunction, double knockouts have an almost complete lack of cartilage (Provot and Schipani, 2005). The replacement cartilage observed during endochondral bone formation varies from persistent cartilage in its expression of type X collagen and this is caused by expression of Runx2, which pushes mesenchymal cells down an osteogenic pathway (de la Fuente and Helms, 2005). Sox9 is required in the early stages of endochondral bone formation but is not required for intramembranous bone formation, presumably due to the lack of cartilage intermediate (de la Fuente and Helms, 2005).

### ***Matrix production***

When mature, chondrocytes produce the cartilaginous matrix. This matrix is required to withstand high levels of cyclic loading without damage to the structure. More than 95% of the volume of cartilage is extracellular matrix, the cellular component is only approximately 3% (Aigner and Stove, 2003). The major contributor to the bulk of cartilage is water, which comprises 70-80% of the wet weight of the tissue (Aigner and Stove, 2003). This provides the cushioning required when force is applied to the cartilage. Collagen provides strength and a degree of rigidity to the matrix, it comprises approximately 15% of the wet weight with the majority being type II collagen (Aigner and Stove, 2003). The other significant



**Figure 1.1: Mesenchymal cell differentiation into chondrocyte and osteoblast lineages.**

Chondrocyte and osteoblast differentiation is governed by a variety of external signals (above arrows) and transcription factors (below arrows). FGF, fibroblast growth factor; BMP, bone morphogenic protein; *Ihh*, Indian hedgehog; *PTHrP*, parathyroid hormone-related peptide; TCF, T cell factor; LEF, lymphoid enhancer binding factor; *Osx*, Osterix; NFATc1, nuclear factor of activated T cells.

components are the proteoglycans, mainly aggrecan; these provide the fluid binding capacity of the matrix and provide it with elasticity (Aigner and Stove, 2003). Cartilage forms the template for the long bones. The removal of cartilage to allow the formation of bone by osteoblasts is performed by osteoclasts, also termed chondroclasts. The cartilage provides the scaffold for bone formation. New bone formation proceeds with the arrival of the vasculature, and is performed by osteoblasts. The method of arrival of these osteoblasts is contentious; circulating precursors might be introduced by the blood vessels (Eghbali-Fatourehchi *et al.*, 2005), or precursors may migrate to the site through breaches in the cartilage mould.

## ***Osteoblasts***

### ***Differentiation***

OB form from pluripotent mesenchymal stem cells through a series of stages involving proliferation, matrix production, and matrix mineralisation (**Figure 1.1**). Differentiation into an osteoblast phenotype is governed the master regulator Runx2 (also known as core binding factor alpha 1 or cbfa1). Runx2 is a runt domain containing transcription factor that is essential for bone formation *in vivo* (Komori *et al.*, 1997; Otto *et al.*, 1997), and osteoblast differentiation *in vitro* (Ducy *et al.*, 1997). It is not sufficient for bone formation, however; unidentified factors in the cartilage anlagen also influence osteogenesis. Runx2 positively regulates the expression of the bone matrix specific genes type I collagen, osteopontin, osteocalcin, bone sialoprotein and fibronectin (Komori, 2003). Runx2 is also important in the process of chondrocyte hypertrophy and vascular invasion (Komori, 2003).

Following Runx2 signalling pre-osteoblasts are further induced to differentiate towards the osteoblast lineage by Osterix (Osx), a downstream target of Runx2 (Kobayashi and Kronenberg, 2005). Osx, a zinc finger containing transcription factor, appears to be responsible for pushing cells towards an osteoblast phenotype as opposed to a chondrocyte phenotype (Nakashima *et al.*, 2002) and its activity is enhanced by direct interaction with nuclear factor of activated T cells (NFATc1) (Koga *et al.*, 2005). The Wnt signalling system has also been implicated in regulation of osteoblast development as knockout of an essential co-receptor for Wnt signal

transduction, LRP5, led to decreased bone formation and osteopenia (Gong *et al.*, 2001).

Osteoblast differentiation leads to the expression of a variety of genes involved in matrix synthesis and also in osteoclastic regulation. The mature osteoblast phenotype is characterised by production of type I collagen, osteocalcin and alkaline phosphatase, leading to extracellular matrix deposition and mineralisation. Osteoblasts also express a variety of molecules that regulate the formation and activity of the bone-resorbing cell, the osteoclast. These factors will be described in detail later. There are three eventual fates for osteoblasts: a small proportion become embedded within the matrix they produce to become osteocytes; other osteoblasts will become the bone lining cells, a flattened layer of cells that line the inactive bone surface; the remainder will undergo apoptosis. Recent evidence points to the bone lining cells, thought to be quiescent osteoblasts, being vital in supporting the most immature haematopoietic precursor cells at the periphery of the marrow (Zhang *et al.*, 2003).

#### *Matrix deposition*

The main function of the osteoblast is to produce the extracellular matrix of bone. The organic, collagenous matrix is produced first and is then subsequently mineralised. These two processes are quite distinct.

#### *Collagen*

Osteoblasts transcribe and secrete predominantly type I collagen; this consists of a triple helix formed from 2 identical  $\alpha 1$  polypeptides (*COL1A1* gene product) and a similar, but distinct,  $\alpha 2$  polypeptide (*COL1A2* gene product). These genes are on separate chromosomes and have different promoter sequences; both are required for successful collagen formation.

Collagen formation proceeds through a series of steps leading from gene transcription to translation and subsequent post-translational processing of the peptides. The *COL1A1* and *COL1A2* promoter sequences contain binding sites for Runx2, and are activated during the differentiation of pre-osteoblasts (Ducy *et al.*,

1997). A variety of other factors are capable of initiating collagen gene transcription; one of the most important of these is TGF beta, which has been implicated in the development of fibrosis (Mauviel, 2005).

The individual peptides are translated into the lumen of the endoplasmic reticulum where they associate through their c-terminal propeptide regions (Prockop, 1990). This allows interaction between the 3 chains and the proximity required for disulfide bond formation by the enzyme protein disulfide isomerase (PDI). This enzyme is a beta subunit of a larger complex that also incorporates the collagen prolyl 4-hydroxylase (P4OH) as the alpha subunit. Following disulfide stabilisation of the structure a number of proline residues (~100) within the chains are hydroxylated by P4OH; this reaction utilises molecular oxygen, 2-oxoglutarate and a co-ordinated  $\text{Fe}^{2+}$ , the antioxidant ascorbic acid 'resets' the P4OH back to its active state following the reaction (Counts *et al.*, 1978). The activity of P4OH is inhibited by molecules that mimic 2-oxoglutarate, and is also affected by changes in iron, oxygen, or vitamin C levels. This proline hydroxylation is essential to allow triple helix formation, the hydroxyproline residues increase the thermal stability of the triple helix such that it is stable at body temperature (Fessler and Fessler, 1974). In the absence of sufficient proline hydroxylation the triple helix is denatured at body temperature and neutral pH. During the process of proline hydroxylation approximately 20 lysine residues are targeted for hydroxylation by several procollagen lysine, 2-oxoglutarate, dioxygenases (PLOD). Like the P4OH, the PLODs are dependent on molecular oxygen for their activity (Kikuchi *et al.*, 1983). Once sufficient proline hydroxylation has occurred the triple helix self assembles in a zipper-like fashion that initiates from the C-terminal propeptide (Prockop, 1990).

Upon successful creation of the triple helix conformation, the procollagen is transported to the Golgi apparatus, where it is packaged into vesicles and transported to the plasma membrane. The procollagen is then secreted into the extracellular space whereupon the propeptides are cleaved by a variety of C and N-terminal proteinases. Removal of the propeptides decreases the solubility of the molecule approximately 2000-fold (Prockop, 1990). All of the information required for fibril formation is contained within the collagen molecule and fibrils self assemble. The mature collagen undergoes further processing by the enzyme lysyl oxidase (LOX). It acts

upon the lysine and hydroxylysine residues to create aldehyde residues, which condense with other lysine and hydroxylysine residues in adjacent molecules to form covalent linkages, this reaction also requires molecular oxygen and results in mature, cross-linked, collagen fibrils (Kagan and Li, 2003). The cross-linking process initiated by LOX continues throughout the lifetime of the collagen. As collagen ages, it becomes increasingly interlinked and loses some of its flexibility; this process has been linked to increased brittleness of connective tissue in arteriosclerosis and is recognised as an inevitable outcome of ageing (Bailey *et al.*, 1998).

### *Mineralisation*

The process of mineralisation of the collagenous matrix is initiated within matrix vesicles in osteoblasts. These vesicles provide a site of nucleation from which the hydroxyapatite crystals form (Balcerzak *et al.*, 2003).  $\text{Ca}^{2+}$  is freely available in the extracellular environment and is transported to the matrix vesicles through  $\text{Ca}^{2+}$  channels. Phosphate is derived from the activity of phosphatases such as alkaline phosphatase (ALP), which produce free phosphate from numerous substrates including adenosine monophosphate (AMP). The activity of ALP can be inhibited by pyrophosphate (Balcerzak *et al.*, 2003). Within the matrix vesicles hydroxyapatite crystals accumulate; when sufficient size has been attained the crystals break the membrane of the vesicles in an as yet undefined manner. Once in the extracellular environment, there is sufficient free  $\text{Ca}^{2+}$  and  $\text{P}_i$  to maintain hydroxyapatite growth into crystalline arrays (Balcerzak *et al.*, 2003). Hydroxyapatite formation results in the production of excess protons. Low pH inhibits the formation of hydroxyapatite crystals; carbonic anhydrase is present in the matrix vesicles to prevent the accumulation of excess acid that would be detrimental to mineralisation (Balcerzak *et al.*, 2003). Hypertrophic chondrocytes also produce matrix vesicles; however, these differ in their composition compared to osteoblast vesicles, and are thought to be a product of dying cells (Balcerzak *et al.*, 2003).

Sites of mineralisation are tightly controlled; mineralisation at ectopic sites is detrimental as it increases the stiffness of the extracellular matrix. Ectopic mineralisation appears to be prevented by the Gla-containing protein, matrix-Gla protein (MGP). The mechanism of this inhibition is not known; however, knockout

mice deficient in MGP have increased bone mineral and ectopic vascular calcification (Luo *et al.*, 1997). Osteocalcin (also termed bone-Gla protein) contains the same Gla modification, but is not thought to be involved in inhibiting mineralisation (Murshed *et al.*, 2004).

## **Modulators of bone development**

Chondrocyte and osteoblast development require the activities of the factors mentioned previously; however a large number of other factors are able to positively and negatively influence bone formation during development. Some of these are briefly reviewed below.

### *FGFs*

FGF signalling is important for normal cartilage and bone formation, as activating mutations in FGFR3 lead to a variety of skeletal dysplasias typified by premature growth plate and cranial suture closure (Provot and Schipani, 2005). FGFR3 activation inhibits chondrocyte proliferation. The primary activating ligands are FGF18 and FGF9; these factors are expressed in the growth plate and *fgf18*<sup>-/-</sup>, and *fgf9*<sup>-/-</sup> animals have a similar phenotype to *fgfr3*<sup>-/-</sup> animals (Ornitz, 2005).

### *BMPs*

The effects of FGFs are offset by the activity of bone morphogenic proteins (BMPs). BMPs are members of the transforming growth factor  $\beta$  superfamily. They act to drive the patterning of skeletal elements, and are positive regulators of chondrocyte proliferation and differentiation (Provot and Schipani, 2005). They were first identified due to their ability to induce bone and cartilage formation at ectopic sites of delivery. BMP -2, -4 and -7 are capable of inducing the differentiation of osteoblasts and chondrocytes from mesenchymal precursors and also act to inhibit the formation of adipocytes (Wan and Cao, 2005). BMPs enhance all stages of chondrocyte differentiation, from commitment to maturation of the chondrogenic phenotype (Wan and Cao, 2005).



### *Indian hedgehog*

Indian hedgehog (Ihh) and parathyroid hormone-related peptide (PTHrP) participate in a feedback loop to regulate long bone development. Ihh is expressed in pre-hypertrophic chondrocytes and is necessary and sufficient to induce PTHrP expression (Wan and Cao, 2005). PTHrP negatively regulates the switch from a proliferating chondrocyte to a mature hypertrophic chondrocyte (Provot and Schipani, 2005). Hence, a negative feedback mechanism exists whereby Ihh induces the production of PTHrP that prevents chondrocyte hypertrophy and subsequently inhibits further production of Ihh. Ihh also directly stimulates chondrocyte proliferation in a PTHrP independent manner (Provot and Schipani, 2005). Together, Ihh and PTHrP regulate the rate of cartilage progression to hypertrophy and ultimately osteogenesis.

### *Osteocytes*

The terminal differentiation state of osteoblasts is the osteocyte; these are the most abundant cell type in mature bone. They form when osteoblasts depositing matrix become embedded within that matrix, leading to gene expression changes that terminate matrix production and prevent further division of the cell. Osteocyte cell processes extend to neighbouring osteocytes and the surface of the bone through an extensive canalicular network. This network provides the largest cell surface area in the body for ion exchange (Knothe Tate *et al.*, 2004). It also allows signals to be passed between cells at the surface of the bone and osteocytes resident deep within the matrix. Osteocytes interact with other cells through gap junctions at sites of cell-to-cell contact (Stains and Civitelli, 2005). Death of osteocytes within their lacunae is thought to lead to matrix removal and engulfment of the cell body by osteoclasts (Gu *et al.*, 2005; Noble and Reeve, 2000); however, it has also been observed that dead osteocytes calcify within their lacunae without evidence of bone remodelling (Boyde, 2003).

Although osteocytes embedded within the bone matrix are relatively quiescent they require a vascular supply to maintain their viability. The osteocytes reside in lacunae deep within the matrix; the distances involved in diffusion the length of these processes to osteocytes deepest within the matrix, and the size of the extracellular

space within the canaliculi are thought to be prohibitive to a diffusion model (Knothe Tate *et al.*, 1998; Wang *et al.*, 2004). Tracer studies to investigate the movement of different sized particles have implicated stress-induced fluid flow in the transport of molecules through the canalicular system (Knothe Tate and Knothe, 2000; Wang *et al.*, 2000). Despite these results arguments still persist that there must be an active transport mechanism within the cells themselves as analysis of fluid flow in osteons indicated that neither passive diffusion nor stress induced fluid flow is sufficient to maintain the viability of osteocytes (Wang *et al.*, 2004). It is likely that all three mechanisms are involved, dependent on the size of the molecule requiring transport.

### *Mechanical loading*

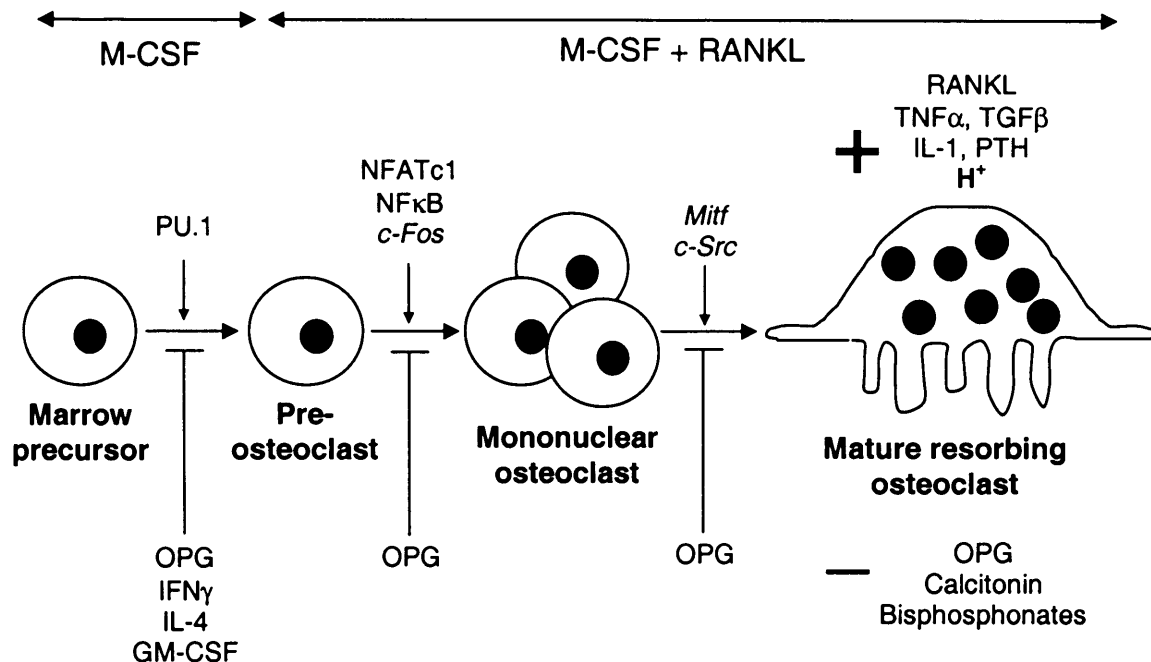
The nutrient requirements of osteocytes make them uniquely positioned to detect mechanical loading to the bone. Fluid flow within canaliculi is generated by compression of the bone matrix induced during loading of the bone, coupled to the hydrostatic pressure created by the circulatory system. It has been proposed that loading induced fluid flow causes shear stresses within osteocyte canaliculi leading to signalling cascades and effector molecule release that ultimately results in new bone formation at the bone surface (Klein-Nulend *et al.*, 1995). Osteocytes acquire their O<sub>2</sub> via the canalicular network; it has been proposed that lack of loading leads to decreased fluid flow and subsequent osteocyte hypoxia resulting in disuse related bone loss (Dodd *et al.*, 1999; Gross *et al.*, 2001). Cyclic loading leads to the release of several effector molecules, which of these are responsible for the effect are yet to be conclusively identified although nitric oxide and prostaglandin E<sub>2</sub> are likely to be involved (Cheng *et al.*, 2001; Zaman *et al.*, 1999).

### *Osteoclasts*

#### *Differentiation*

OC are specialised bone resorbing cells derived from haematopoietic cells of the monocyte-macrophage lineage under the influence of macrophage colony stimulating factor (M-CSF) and receptor activator of NFκB ligand (RANKL); these factors are essential and sufficient for osteoclast formation (Cecchini *et al.*, 1997; Lacey *et al.*, 1998). Bone formation and resorption occur in cycles in normal individuals, and

osteoblasts are able to control the formation and activity of osteoclasts through expression of these two essential factors.



**Figure 1.2: Pathway of osteoclast differentiation and important mediators.**

Above arrows indicate positive regulators of osteoclast differentiation, + indicates positive regulators of osteoclast activity. Below (blunt-ended) arrows indicate negative regulators of osteoclast differentiation, - indicates negative regulators of osteoclast activity. **Abbreviations:** M-CSF, macrophage-colony stimulating factor; RANKL, receptor activator of NFκB ligand; NFATc1, nuclear factor of activated T cells; NFκB, nuclear factor κB; OPG, osteoprotegerin; IFN, interferon; IL, interleukin; GM-CSF, granulocyte macrophage-colony stimulating factor; TNF, tumour necrosis factor; TGF, transforming growth factor; PTH, parathyroid hormone.

#### *Macrophage colony stimulating factor (M-CSF)*

OC differentiate from monocytic precursors through a well-characterised series of stages (**Figure 1.2**). One of the earliest signals required to begin the progression from a marrow precursor into an osteoclast is M-CSF; this is produced by stromal cells including the osteoblast. Activation of c-fms, a tyrosine kinase linked receptor, by M-CSF binding leads to the expression of transcription factors such as *c-fos* (Blair

*et al.*, 2005; Yao *et al.*, 2005). M-CSF is also required for macrophage differentiation, the *op/op* mouse, which has an inactivating mutation in the CSF-1 gene, fails to form either osteoclasts or macrophages (Stanley *et al.*, 1997). The biology of M-CSF is complicated by the presence of various different forms. Osteoblasts express and produce a proteoglycan linked form that is sequestered in the extracellular matrix, as well as a membrane bound form, it is thought that these restrict the influence of M-CSF to the local environment (Stanley *et al.*, 1997). M-CSF does not appear to be involved in the activation of mature osteoclasts to resorb bone, it merely acts to induce proliferation and differentiation, although there is some disagreement in this area due to the observation that M-CSF binding leads to activation of *Src* kinase, an essential mediator of osteoclast activity (Tanaka *et al.*, 2003).

#### *Receptor activator of NF $\kappa$ B ligand (RANKL)*

The other essential cytokine for osteoclast differentiation is RANKL. RANKL was initially identified as a T cell derived cytokine essential for dendritic cell proliferation and survival (Anderson *et al.*, 1997). RANKL is a member of the TNF superfamily that is the ligand for RANK and the soluble decoy receptor, osteoprotegerin (OPG). This trinity of molecules exists in a delicate balance to control osteoclast formation and activity. It was known that osteoblasts were required for osteoclast formation but the exact interaction between these cells was not clear (Hattersley and Chambers, 1989). In the late 90's several publications brought to light the existence of osteoblast derived OPG and its ligand RANKL as key regulators of osteoclast formation and activity (Bucay *et al.*, 1998; Burgess *et al.*, 1999; Fuller *et al.*, 1998; Lacey *et al.*, 1998; Yasuda *et al.*, 1998). This was a major development in bone biology as the production of recombinant RANKL allowed the study of osteoclast formation *in vitro* independent of osteoblast / stromal cell interference.

RANKL is produced by osteoblasts in response to a range of osteoclastogenic stimuli such as parathyroid hormone (PTH), PGE<sub>2</sub> or vitamin D<sub>3</sub>. The production of RANKL is counteracted by OPG production, which binds free RANKL, sequestering it from binding to RANK on osteoclasts. Mice overexpressing OPG have an

osteopetrotic phenotype, whereas mice lacking OPG have an osteoporotic phenotype, serum levels of OPG correlate with bone turnover markers (Kong *et al.*, 1999). Mice deficient in RANKL have major skeletal defects accompanied by failed tooth eruption and severe osteopetrosis. They exhibit small, club shaped long bones, the bone marrow spaces are nearly completely filled with cartilage and bone matrix, and they have a complete absence of TRAP positive osteoclasts (Kong *et al.*, 1999).

RANKL binding to its receptor RANK on M-CSF-stimulated monocytes leads to the expression of a wide variety of osteoclast specific genes including cathepsin K and TRAP (Day *et al.*, 2004). A wide variety of transcription factors have been implicated in signal transduction from the receptor to the genome; the key factors are discussed below.

#### *Intracellular signalling pathways*

A variety of transcription factors have been implicated in relaying differentiation and activation signals from the osteoclast surface to initiate gene expression. These have generally been identified due to bone defects in knockout animals.

#### *PU.1*

PU.1 knockout mice are osteoclast deficient, accompanied by deficient haematopoiesis, indicating that PU.1 is essential for the early stages of osteoclast differentiation (Tondravi *et al.*, 1997). The general disruption of haematopoiesis is a common finding in knockout animals with defects in the early stages of osteoclast differentiation because many of the pathways are shared at these early stages with other haematopoietic cell lineages. The role of PU.1 in osteoclast differentiation has been linked to the induction of expression of c-fms and RANK in osteoclast precursors (Blair *et al.*, 2005; Kwon *et al.*, 2005).

#### *TRAF6*

The binding of RANKL to its receptor RANK initiates a signal transduction cascade involving a multitude of intermediates. The major pathways identified involve signalling through TNF receptor associated factors (TRAFs). RANK has binding sites for TRAF 1, 2, 3, 5 and 6 (Feng, 2005). Of the TRAF proteins, the

interaction with TRAF 6 appears to be most important, as TRAF6<sup>-/-</sup> mice develop severe osteopetrosis due to a lack of osteoclasts (Lomaga *et al.*, 1999). TRAF6 binding leads to activation of NFκB, and the three mitogen activated protein kinases (MAPK) JNK, ERK and p38 (Feng, 2005). However, TRAF binding is not unique to RANK; it has been reported that the inflammatory cytokines TNFα and IL-1 are capable of inducing osteoclast formation in the absence of RANKL. Receptors for these cytokines belong to the TNF superfamily and ligand binding activates various TRAFs. Although both of these cytokines are capable of enhancing RANKL-induced osteoclast formation and activity, neither can induce osteoclastogenesis alone (O' Gradaigh *et al.*, 2004); a further signalling pathway must be induced by binding of RANKL to its receptor.

### *NFκB*

NFκB is a transcription factor associated with stress and immune responses, and it exists as a dimer of highly structurally similar proteins. It is held in the cytoplasm by inhibitor of κB (IκB), and is released upon activation of IκB kinase (IKK) to translocate to the nucleus (Franzoso *et al.*, 1997). As the proteins constituting the NFκB dimer are highly similar, there is a degree of redundancy when knockouts of individual subunits are made. However, double knockouts of subunits p50 and p52 developed osteopetrosis due to defective osteoclast development, indicating the essential nature of this factor for osteoclastogenesis (Franzoso *et al.*, 1997). These mice also had defects in B cell development, again highlighting the shared pathways between osteoclasts and their immune system counterparts (Franzoso *et al.*, 1997).

NFκB was the initial transcription factor identified as being involved in osteoclast formation; however, there is increasing evidence that the three MAPK pathways (JNK, ERK and p38) are mediating effects on osteoclastogenesis induced by other TNF superfamily members. TNFα-induced osteoclast formation requires the phosphorylation of JNK (Kanazawa and Kudo, 2005), and TNFα-induced osteoclast survival requires the phosphorylation of ERK (Lee *et al.*, 2001). Interference with osteoclast formation by inhibitors such as TNF receptor apoptosis inducing ligand

(TRAIL) occurs via the p38 pathway and has no impact upon NFκB signalling (Blair *et al.*, 2005).

### *NFATc1*

Calcium signalling induces the activity of the phosphatase calcineurin, which stabilises cytoplasmic nuclear factor of activated T cells (NFATc) proteins and translocates them to the nucleus (Crabtree and Olson, 2002). DNA binding leads to the transcription of target genes, amongst them is NFATc1 itself, a form of auto-amplification of the signal. Signalling is prevented by the action of kinases such as GSK3, which prevent nuclear translocation of the NFAT proteins (Crabtree and Olson, 2002). NFATc1 was the transcription factor gene most strongly induced upon RANKL binding to RANK (Takayanagi *et al.*, 2002). Furthermore, embryonic cells lacking NFATc1 were unable to differentiate into osteoclasts, and embryonic cells overexpressing NFATc1 differentiate into osteoclasts spontaneously without the requirement for RANKL (Takayanagi, 2005). NFATc1 is also induced by NFκB, and has the capacity to link all of the signalling pathways that have been associated with osteoclastogenesis (Takayanagi *et al.*, 2002). These results suggest that NFATc1 could be a master regulator of osteoclastogenesis.

### *c-Fos / AP1*

*c-Fos* was initially identified as being required for osteoclastogenesis by the osteopetrotic phenotype of the knockout mouse. It forms homo- or heterodimers with members of the Jun family or ATF family of transcription factors to form the dimeric transcription factor AP1. The *c-fos* knockout was of particular interest because osteoclast formation was severely affected whilst macrophage differentiation was not (Grigoriadis *et al.*, 1994). The absence of osteoclasts was coupled with a large increase in macrophage number, which indicated that *c-fos* might act as the switch between macrophage and osteoclast lineages (Grigoriadis *et al.*, 1994). Knockout studies on other constituents of the AP1 transcription factor complex revealed diverse effects on both osteoblasts and osteoclasts. *c-Jun* knockouts are embryonically lethal; however, macrophage lineage specific knockouts showed a severe osteopenia due to cell autonomous defects in both osteoclast and osteoblast development (Kenner *et al.*,

2004). These studies revealed a more complex role for the AP1 transcription factor in bone biology.

#### *Microphthalmia-associated transcription factor (Mitf)*

*Mitf* was identified as important for osteoclast formation and activity due to the osteopetrotic phenotype of *Mitf*<sup>-/-</sup> mice. These mice had short stature and radio-opaque bones, the typical signs of osteopetrosis (Hershey and Fisher, 2004). Examination of the bones revealed the presence of TRAP positive osteoclasts but these were mainly mononuclear, and resorption was severely disrupted (Hershey and Fisher, 2004). Further examination indicated that *Mitf* was most likely involved in regulating the fusion of osteoclast precursors. *Mitf* has been shown to interact synergistically with PU.1 to induce transcription of the osteoclast specific marker TRAP (Hershey and Fisher, 2004). *Mitf* also induces the anti-apoptotic factor Bcl-2, which is important for the survival of the osteoclast lineage cells (Takayanagi, 2005).

#### *c-Src*

*c-Src* was identified as an essential osteoclast activator by the osteopetrotic phenotype of the knockout animal (Soriano *et al.*, 1991). *c-Src* is involved in cytoskeletal rearrangement, cell motility and regulation of gene expression in response to integrin adhesion (Shattil, 2005). These responses are essential for the process of bone resorption, and *c-Src* knockout animals show severe osteopetrosis associated with a defect in osteoclast activation (Miyazaki *et al.*, 2004). Osteoclasts normally express high levels of *Src* protein; the defect in *src*<sup>-/-</sup> animals was specific to osteoclasts and was cell-autonomous (Lowe *et al.*, 1993). *Src* plays an important role in the activation of osteoclasts upon  $\alpha_v\beta_3$  integrin binding through its interaction with Pyk2 and c-Cbl (Sanjay *et al.*, 2001).

### ***Bone resorption***

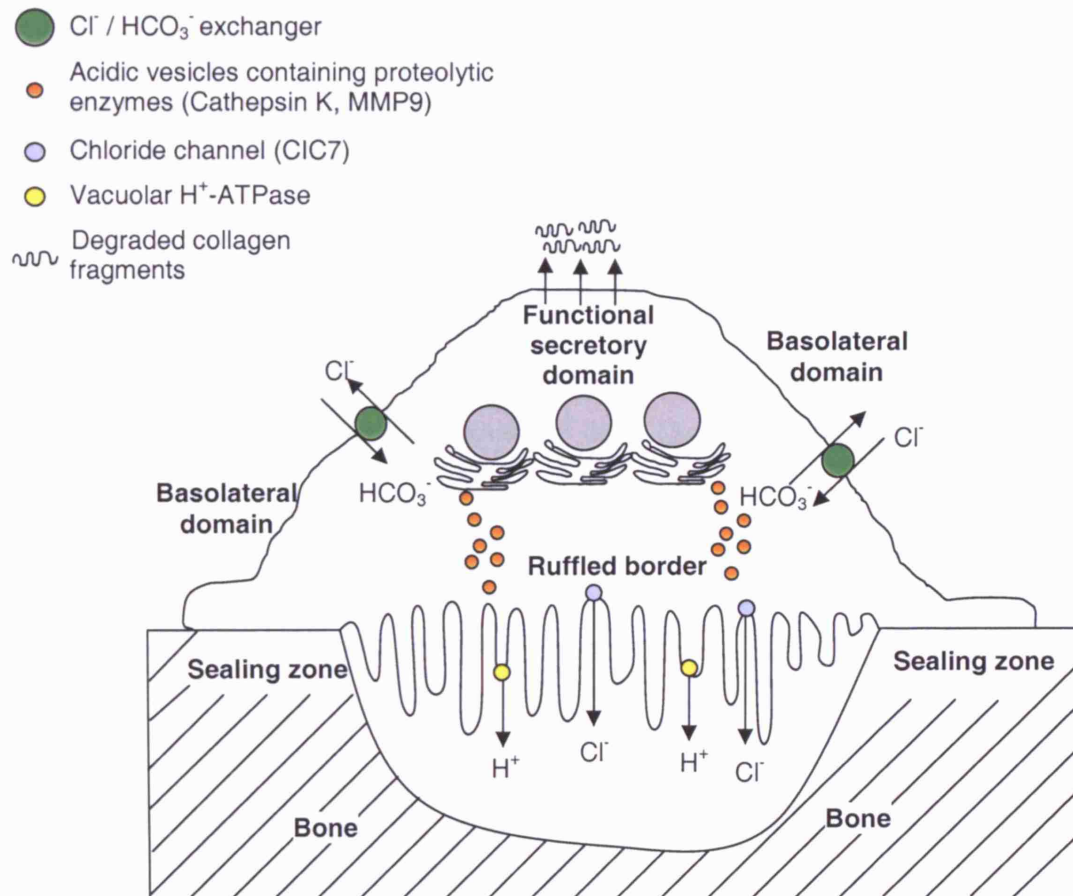
Bone resorption is the removal of bone mineral and matrix through a series of physicochemical and enzymatic processes. The initial step in osteoclast activation is polarisation of the cell body into 4 distinct membrane regions: the sealing zone and ruffled border adjacent to the bone matrix, and the basolateral domain and functional



secretory domain facing away from the bone surface (**Figure 1.3**) (Mulari *et al.*, 2003). The sealing zone is a ring structure tightly attached to the bone surface, which encircles the area of plasma membrane comprising the ruffled border. The sealing zone is typified by dense accumulations of filamentous actin, which form a typical ring structure when imaged using confocal microscopy (Mulari *et al.*, 2003). The sealing zone acts to create a microenvironment suitable for the removal of bone collagen and mineral without exposing other cells to the hostile environment within the resorption lacuna, it also acts to concentrate the required bone degrading enzymes and protons to a localised area (Mulari *et al.*, 2003).

The attachment between the bone surface and the osteoclast is regulated initially by integrins. The vitronectin receptor (VNR), composed of  $\alpha v \beta 3$  integrins, regulates the initial attachment to non-collagenous proteins in the bony matrix, whereas  $\alpha 2 \beta 1$ , binds to collagen (Horton, 1995). Blockade of these receptors using decoy ligands leads to detachment of osteoclasts and inhibition of resorption (Helfrich *et al.*, 1996). Following attachment these proteins are not present in the sealing zone; the proteins that control attachment to the bone during resorption are not clear.

The ruffled border is essential to bone resorption as it forms the active resorption site. Once attachment to the surface has been accomplished, fusing of acidic vesicles with the ruffled border membrane acidifies the resorption pit. These vesicles contain proteolytic enzymes such as cathepsin K and matrix metalloproteinase 9 (MMP9) in addition to hydrochloric acid (Mulari *et al.*, 2003). Removal of the inorganic phase of bone is required before degradation and removal of collagen can be performed. The hydroxyapatite is dissolved by protons pumped through the ruffled border by vacuolar  $H^+$ -ATPase into the resorption lacuna; charge is balanced by pumping of  $Cl^-$  from the cytoplasm into the resorption pit, and intracellular pH is maintained by a  $Cl^- / HCO_3^-$  exchanger in the basolateral domain (Teitelbaum, 2000). The concerted activity of these pumps and exchangers results in acidification of the resorption pit to pH 4.5, without any change in osteoclastic intracellular pH. The supply of protons and bicarbonate ions is generated by carbonic anhydrase, which is highly expressed in osteoclasts. Following dissolution of mineral and degradation of collagen in the resorption lacuna, peptide fragments are endocytosed by the osteoclast and



**Figure 1.3: Schematic of a resorbing osteoclast.**

Mature osteoclasts initiate bone resorption by forming a sealed compartment against the bone matrix via integrin adhesions in the sealing zone. Protons and chloride ions are pumped into the resorption pit through the ruffled border, chloride maintains neutrality whilst intracellular pH is maintained by exchanging bicarbonate with chloride in the extracellular space. Acidic lysosomal vesicles are produced in the Golgi and transported to the ruffled border where they fuse and release their cargo of proteolytic enzymes into the resorption pit. Protein degradation products are transported via endocytosis through the ruffled border and expelled from the functional secretory domain into the extracellular space.

transported through the cell to the functional secretory domain where they are expelled into the extracellular space (Mulari *et al.*, 2003).

### *Cathepsin K*

The organic phase of bone is removed by the action of cathepsin K and other proteases following mineral dissolution. Cathepsin K is a cysteine protease present within lysosomal vesicles, the ruffled border and resorption lacuna of resorbing osteoclasts that is active at low pH, and inactive at neutral or alkaline pH (Goto *et al.*, 2003). It targets both telopeptide and helical regions of type 1 collagen, whereas interstitial collagenase is only capable of cleaving collagen in the helical region (Garnero *et al.*, 1998). Mice lacking this enzyme are osteopetrotic due to deficient degradation of the organic component of bone (Saftig *et al.*, 1998). Cathepsin K deficient mice are capable of some resorption and compensate for the lack of cathepsin K by increased formation of osteoclasts. They also increase expression of other bone degrading enzymes such as cathepsin L, tartrate resistant acid phosphatase (TRAP), MMP9, MMP13 and MMP14 (Kiviranta *et al.*, 2005).

### *TRAP*

OC express high levels of TRAP and its role in osteoclast biology has been difficult to determine. The active site of TRAP contains a binuclear iron centre with 2 ferric ions, one of which is redox-active, allowing the production of reactive oxygen species (Vaaraniemi *et al.*, 2004); it also has phosphatase activity and can dephosphorylate osteopontin and bone sialoprotein with maximal activity at pH 5.7 (Allen *et al.*, 1989). TRAP<sup>-/-</sup> animals develop mild osteopetrosis associated with decreased osteoclastic resorption but unaltered osteoclastogenesis (Hayman *et al.*, 1996; Hollberg *et al.*, 2002). Osteoclasts from these animals have disturbed intracellular trafficking and defects in collagen processing following degradation in the resorption lacuna. TRAP has been proposed to produce reactive oxygen species that aid in the degradation of collagen fragments endocytosed from the resorption pit (Hollberg *et al.*, 2002).

## *MMPs*

The role of MMP9 in osteoclastic resorption is unclear. Osteoclasts express high levels of MMP9; however, MMP9<sup>-/-</sup> mice have a relatively normal adult skeleton (Vu *et al.*, 1998). The defects observed in MMP9<sup>-/-</sup> mice all relate to impaired growth plate vascular invasion, leading to elongated growth plates; this defect is eventually overcome (Vu *et al.*, 1998). The angiogenic response to tissue ischaemia is also dependent upon the presence of MMP9 in macrophages (Johnson *et al.*, 2004); the effects of loss of osteoclast-derived MMP9 on the growth plate are likely to be due to the failure of vascularisation rather than resorption defects.

## ***Basic multicellular unit***

Bone mineral and matrix are removed and replaced as a form of maintenance. This procedure requires a coordinated effort amongst the various cell types in bone; the basic multicellular unit (BMU) is the term used to describe this cohort of cells. The BMU consists of osteoclasts removing bone, osteoblasts following in their wake to replace the bone, and the capillary that services their requirements for oxygen and nutrients and removal of waste products (Frost, 1998). The intimate link between the cells within the BMU allows for the sensitive control of bone remodelling. Bone formation is linked to bone resorption via the osteoblastic production of osteoclast-modulating factors such as RANKL, OPG, M-CSF and protons. Many factors are able to regulate bone turnover through both direct and indirect effects on osteoblasts and osteoclasts, several of these are discussed in detail below.

## **Factors affecting bone turnover**

### ***Cytokines***

Inflammation is associated with the production of cytokines and localised hypoxia (Peyssonaux and Johnson, 2004). Stromal cells and cells of the immune system, both of which are in close proximity to the bone cells, can produce cytokines that positively and negatively regulate bone cell activity. Several of the key cytokines are mentioned below.

### *Tumour necrosis factor (TNF $\alpha$ )*

RANK, RANKL and OPG are members of the TNF superfamily, sharing many of the intracellular signalling pathways with other members. TNF $\alpha$ , the first family member to be discovered, is produced mainly by activated macrophages, and is thought to be the main osteoclastogenic cytokine produced during inflammation (Kwan Tat *et al.*, 2004). TNF $\alpha$  is able to stimulate osteoclast formation, although it requires the presence of permissive levels of RANKL to do so (Lader and Flanagan, 1998; Lam *et al.*, 2000); it also promotes survival of mature osteoclasts (Lee *et al.*, 2001). It has also been shown to enhance osteoclastic resorption through osteoblast-dependent mechanisms (Thomson *et al.*, 1987). TNF $\alpha$  is capable of inducing osteoclast formation in the absence of RANKL, but IL-1 is also required for this effect to occur (Kudo *et al.*, 2002). The effects of TNF $\alpha$  on osteoclasts are mediated through TRAF2 and, ultimately, NF $\kappa$ B activation (Kanazawa and Kudo, 2005).

### *Interleukins*

A variety of interleukins have been implicated in both positive and negative regulation of bone formation. The main pro-resorptive interleukins are IL-1 and IL-6. IL-1 binding to the IL-1 receptor leads to signal transduction via TRAF 6, and NF $\kappa$ B (Kwan Tat *et al.*, 2004). IL-1 is a potent inducer of TNF $\alpha$ , and TNF $\alpha$  is likewise a potent inducer of IL-1, so these cytokines act synergistically in a positive feedback loop and are thought to be the main cause of bone destruction in rheumatoid arthritis (Schiff, 2000). IL-1 enhances RANKL-induced osteoclast formation (Ma *et al.*, 2004). Furthermore, addition of interleukin 1 receptor antagonising protein (IRAP), a soluble inhibitor of IL-1, significantly reduced RANKL induced osteoclast formation in cultures not supplemented with IL-1, indicating that RANKL signalling induces endogenous IL-1 expression (Lee *et al.*, 2005). Recent work also suggests that IL receptor signalling in the hypothalamus may mediate bone mass through central control mechanisms not involving sex steroid pathways (Bajayo *et al.*, 2005). The effect of IL-1 on osteoblasts is to increase the production of M-CSF and PGE<sub>2</sub> whilst decreasing OPG, which would lead to increased osteoclastogenesis (Tanabe *et al.*, 2005).

IL-6 is another major interleukin thought to be involved in bone loss. It signals through the IL-6 receptor and gp130 to activate members of the signal transducers and activators of transcription (STAT) family of transcription factors. Osteoclast formation was stimulated directly by IL-6 in cultures of human peripheral blood mononuclear cells, even in the absence of RANKL (Kudo *et al.*, 2003). IL-6 induces IL-1 expression in osteoblasts leading to increased osteoclast formation (Kwan Tat *et al.*, 2004). IL-6 is also capable of increasing the expression of M-CSF and RANKL in stromal cells (Gorny *et al.*, 2004; Nakashima *et al.*, 2000).

Several interleukins have been identified as inhibiting osteoclast differentiation. Activated T cells produce IL-4, IL-10 and IL-13, which all have inhibitory actions on osteoclast formation (Shinoda *et al.*, 2003). IL-18 and IL-12 produced by stromal cells and lymphocytes also have strong inhibitory actions on osteoclastogenesis (Udagawa, 2003). IL-4 inhibits osteoclast differentiation by interfering with NF $\kappa$ B activation induced via RANKL and TNF pathways, it has no effects on the M-CSF pathway and the inhibition is reversible (Wei *et al.*, 2002). Other interleukins act by inducing interferon  $\gamma$  or GM-CSF production in stromal cells or T cells (Nagata *et al.*, 2003; Shinoda *et al.*, 2003).

#### *Interferon $\gamma$ (IFN $\gamma$ ) / GM-CSF*

IFN $\gamma$  and GM-CSF are produced by activated T cells and are negative regulators of osteoclastogenesis. IFN $\gamma$  rapidly induces the degradation of TRAF6, terminating signalling from RANK on the osteoclast membrane (Takayanagi *et al.*, 2002). GM-CSF inhibits osteoclast differentiation through binding with its receptor on the membranes of pre-osteoclasts (Udagawa, 2003). These factors are involved in maintaining the balance between osteoclast formation and macrophage formation.

#### *TGF $\beta$*

TGF $\beta$  is one of the most abundant non-collagenous proteins in bone matrix. It is sequestered in the bone matrix and released upon bone resorption (Janssens *et al.*, 2005). The role of TGF $\beta$  in regulating bone turnover is complicated. It has been shown to increase the formation of osteoclasts from mononuclear cells in the absence

of osteoblasts, and it has also been shown to decrease formation of osteoclasts in co culture (Quinn *et al.*, 2001). The indirect inhibitory effects of TGF $\beta$  on osteoclasts are mediated by decreased expression of RANKL on osteoblasts combined with an increase in OPG production (Quinn *et al.*, 2001). Its direct effects on osteoclasts are mediated through binding of TGF $\beta$  to its receptor and are synergistic with RANKL-mediated osteoclastogenesis; TGF $\beta$  has also been shown to enhance survival of osteoclasts (Fuller *et al.*, 2000).

TGF $\beta$  also has stimulatory actions on bone formation by osteoblasts. It induces chemotaxis of osteoblast precursors, proliferation and extracellular matrix synthesis (Janssens *et al.*, 2005). The stimulatory effects of TGF $\beta$  on osteoblasts are dependent on the stage of cell differentiation; at later stages, such as during matrix mineralisation, TGF $\beta$  has inhibitory effects (Janssens *et al.*, 2005).

#### *Vascular endothelial growth factor (VEGF)*

VEGF is the major cytokine involved in stimulating angiogenesis, and is essential for bone formation, since vascular invasion is an essential step in endochondral ossification and fracture healing. In addition to its well-documented role in stimulating proliferation and survival of endothelial cells, evidence is emerging that VEGF has diverse, direct effects on bone cells. Both osteoblasts and osteoclasts express VEGF receptors, and are capable of responding to VEGF (Tombran-Tink and Barnstable, 2004). Loss of VEGF leads to impaired endochondral bone formation (Maes *et al.*, 2002; Zelzer *et al.*, 2002). VEGF is required for the vascular invasion of the cartilage anlagen and the delivery of osteoclast and osteoblast precursors to sites of ossification as well as inducing differentiation of chondrocytes and osteoblasts (Carlevaro *et al.*, 2000; Zelzer *et al.*, 2002). Non-union fractures are associated with deficient angiogenesis, indicating that VEGF is required for successful fracture healing (Street *et al.*, 2002). Expression of VEGF by osteoblasts leads to vascular sprouting in vitro and is associated with increased vessel density at sites of fracture (Mayer *et al.*, 2005). Addition of VEGF to osteoblast cultures leads to increased bone nodule formation in vitro and increased alkaline phosphatase activity (Street *et al.*, 2002). VEGF is capable of inducing osteoclast formation in substitution for M-CSF in the osteopetrotic (op/op) mouse via a mechanism not yet understood (Niida *et*

*al.*, 1999). VEGF also has direct effects on osteoclasts. Addition of VEGF to purified, mature rabbit osteoclast cultures led to enhancement of bone resorption and osteoclastic survival (Nakagawa *et al.*, 2000). Osteoclast differentiation is also increased by exposure to VEGF signalling through VEGFR1 (Aldridge *et al.*, 2005).

### *Wnts*

Wnts were identified as being important regulators of bone mass when two human diseases associated with bone disorders were identified as having mutations in LRP5, the co-receptor for Wnt proteins (Westendorf *et al.*, 2004). Wnts signal through 3 separate intracellular pathways, of which the canonical pathway is associated with LRP5. Binding of Wnts to LRP5/LRP6 and Frizzled transmembrane protein complexes initiates a signalling cascade that results in stabilisation of  $\beta$ -catenin and induction of Wnt specific gene expression. Soluble antagonists such as Dickkopf, which competes for receptor complex binding with Wnt, tightly control the activity of Wnt proteins. LRP5 is expressed on osteoblasts and disruption of the gene encoding LRP5 leads to osteoporosis syndromes in humans (Westendorf *et al.*, 2004). Surprisingly, high bone mass phenotypes have also been mapped to mutations in LRP5, these mutations appears to cause decreased affinity of the LRP5 for Dickkopf proteins whilst Wnt binding is unaffected (Westendorf *et al.*, 2004). Wnt proteins act as survival factors for osteoblasts via canonical and non-canonical pathways (Almeida *et al.*, 2005). Signalling via the canonical Wnt pathway leads to expression of Runx2 and subsequent osteogenic differentiation (Gaur *et al.*, 2005). Conversely, Wnt signalling is inhibitory to osteoclastogenesis by causing decreased RANKL production in osteoblasts (Spencer *et al.*, 2006).

### *Sex steroids*

Sex steroids play a vital role in skeletal homeostasis, in both males and females. Their effects are evolutionarily conserved and are related to the requirement for calcium during reproduction (Syed and Khosla, 2005). Oestrogen has long been linked to bone mass; ovariectomy induces bone loss in animal models, and the menopause is associated with development of osteoporosis in women. Oestrogen acts through the oestrogen receptors (ER)  $\alpha$  and  $\beta$ ; osteoblasts express both receptors



(Bonnelye and Aubin, 2002) and are capable of responding directly to oestrogen. Oestrogen receptors have also been identified on osteoclasts; however, many of the effects of oestrogen on osteoclasts are thought to proceed through osteoblasts (Troen, 2003).

Oestrogen withdrawal is associated with increased bone turnover, with osteoclast formation and activity upregulated (Syed and Khosla, 2005). Osteoblast activity is also increased but less so relative to resorption leading to net bone loss. Bone loss is most severe in the trabecular bone, however cortical bone is also affected albeit at a slower rate (Syed and Khosla, 2005). Oestrogen has a bone protective phenotype, it stimulates osteoblasts to proliferate, differentiate and produce bone matrix (Qu *et al.*, 1998). Oestrogen also induces osteoblasts to increase expression of OPG and subsequently inhibit osteoclast formation (Hofbauer *et al.*, 1999; Bord *et al.*, 2003). Treatment of bone marrow cultures, absent of stromal cells, with 17-beta-oestradiol revealed that oestrogen inhibits osteoclast formation by suppressing M-CSF production (Sarma *et al.*, 1998); however, 17-beta-oestradiol had no effect on the activity of mature osteoclasts (Arnett *et al.*, 1996).

Postmenopausal women have an increased risk for atherosclerosis than either premenopausal women or postmenopausal women receiving oestrogen therapy, indicating that oestrogen has protective effects on the cardiovascular system (Volterrani *et al.*, 1995; Barengolts *et al.*, 1998; Mendelsohn and Karas, 1999). Oestrogen increases vasodilation by stimulating nitric oxide production through endothelial nitric oxide synthase (eNOS) (Mendelsohn, 2000). Testosterone is the precursor to oestrogen; it is converted to oestrogen by aromatase. The effects of testosterone on bone metabolism appear to be less clear-cut than those of oestrogen, loss of testosterone only accounted for 30% of bone loss in males whose endogenous sex steroid production was inhibited; the majority of bone effects in males are controlled by oestrogen (Syed and Khosla, 2005).

### ***Calcium homeostasis, vitamin D and PTH***

Ca<sup>2+</sup> is a versatile intracellular signal that regulates many different cellular functions. Blood ionised calcium levels are maintained within a tight range *in vivo*,

between 1.1 and 1.3 mM (Purroy and Spurr, 2002). Systemic calcium levels are controlled by a sensitive multi-organ system involving parathyroid hormone (PTH) and 1, 25 dihydroxyvitamin D<sub>3</sub> (vitamin D<sub>3</sub>). The chief cells of the parathyroid gland respond to decreases in serum calcium by releasing PTH. This then stimulates the hydroxylation of 25 hydroxyvitamin D<sub>3</sub> to its active form 1, 25 dihydroxyvitamin D<sub>3</sub>. Both PTH and vitamin D<sub>3</sub> then act to stimulate calcium reabsorption in the renal tubules and liver, and to stimulate bone resorption and the release of calcium stores from the bone. When calcium levels reach the target of 1.3 mM, a negative feedback mechanism cuts in which terminates PTH release and, subsequently, vitamin D<sub>3</sub> production (Purroy and Spurr, 2002). Calcium release from bone reserves requires the stimulation of bone resorption by osteoclasts; PTH and vitamin D<sub>3</sub> primarily act on osteoblasts to produce RANKL and stimulate osteoclast activity (Udagawa *et al.*, 1999), although evidence is accumulating that there are also direct effects of PTH on osteoclasts (Dempster *et al.*, 2005). PTH also induces rapid acid efflux from osteoblast-like cells (Barrett *et al.*, 1997); this may mediate its pro-resorptive activity, as acidosis activates osteoclasts (discussed in detail below).

### ***Insulin-like growth factors (IGFs)***

The IGF system consists of 2 peptide hormones IGF1 and IGF2, 2 receptors IGF1R and IGF2R, and a variety of binding proteins and proteases. IGF1 and 2 have sequence similarity to insulin and are capable of binding to the insulin receptor (IR) (Denley *et al.*, 2005). The major site of production of IGF1 is the liver and this is controlled by growth hormone (GH) (Dupont and Holzenberger, 2003). IGF2 is produced by a variety of tissues throughout life and is not controlled by growth hormone. The IGF proteins are both important in regulating foetal growth, however most of the functions of IGF2 can be compensated for by IGF1 in adulthood (Dupont and Holzenberger, 2003).

IGF1<sup>-/-</sup> mice are 40% smaller than wild type littermates and most die shortly after birth; on the contrary, although IGF2<sup>-/-</sup> mice are 60% smaller than wild type littermates they have normal growth rates after birth (Dupont and Holzenberger, 2003). The ability of IGF proteins to control body growth leads to obvious

implications for the skeleton. IGF stimulates osteoblast proliferation, differentiation and matrix production, and has a role in regulating osteoblast survival (Zhang *et al.*, 2002). Osteoblasts produce IGF and osteoclast formation is enhanced by addition of IGF to the culture medium (Fukuoka *et al.*, 2005). Another important function of IGF is its ability to induce acid efflux from osteoblastic cells (Santhanagopal and Dixon, 1999), as protons are a potent activator of osteoclast resorption (discussed in detail below).

### ***Leptin***

Leptin is a hormone produced by adipocytes that controls energy regulation. The study by Ducky *et al.* (2000) found that mice deficient in leptin (*ob/ob*) or its receptor (*db/db*) have a phenotype of high bone mass that is not dependent on hypogonadism, hypercortisolism or obesity associated with leptin deficiency (Ducky *et al.*, 2000). Contrary to the findings of Ducky *et al.* are those of Cornish and colleagues (2002), who found that addition of leptin to osteoblast bone forming cultures led to increased bone formation, and that functional leptin receptor was present on osteoblasts. Leptin also inhibited osteoclastogenesis but had no effect on osteoclast activity. Systemic administration of leptin decreased bone fragility and reduced fracture incidence (Cornish *et al.*, 2002). These results confirmed previous findings that leptin has a direct anabolic effect on osteoblasts (Gordeladze *et al.*, 2002; Reseland *et al.*, 2001).

Leptin is not the only hormone controlling food intake and energy metabolism that has also been associated with control of bone turnover. Several gut hormones have now been identified that can regulate bone mass including ghrelin (Fukushima *et al.*, 2005) and amylin (Cornish *et al.*, 1995; Cornish *et al.*, 1998). The link between bone mass and adiposity is a rapidly expanding area of bone research. Both fat mass and bone mass also relate directly to immune status and links between these three areas are becoming clearer.

### ***Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)***

PGE<sub>2</sub> is a vasodilator that is synthesised from arachidonic acid in bone cells via cyclooxygenase 2 (COX2) and released into the local environment (Raisz, 1999). Binding of PGE<sub>2</sub> to EP receptors on bone cells leads to effector functions (Raisz,

1999). PGE<sub>2</sub> has been identified as a potent stimulator of osteoclast formation and resorption (Lader and Flanagan, 1998). However, it has also been observed that PGE<sub>2</sub> inhibits the activity of rodent osteoclasts directly (Collins and Chambers, 1991), and that PGE<sub>2</sub> is a potent inhibitor of osteoclast formation in cultures of CD14<sup>+</sup> cells (Take *et al.*, 2005). The role of osteoblasts in controlling osteoclast responses may account for these differing results (Udagawa *et al.*, 1999). Deletion of COX2 leads to severe impairment of osteoclastogenesis and many factors that induce osteoclast formation act via increasing activity of COX2 and subsequent PGE<sub>2</sub> production in osteoblasts (Raisz, 1999). PGE<sub>2</sub> has also been implicated in mechanotransduction; it induces the expression of COX2 in osteoblasts in a positive feedback loop leading to amplification of signals received from osteocytes within the bone matrix (Pilbeam *et al.*, 1995).

### ***Nitric oxide***

Nitric oxide (NO) is a gas and a free radical, and is a potent vasodilator (Moncada *et al.*, 1988). It is synthesised from L-arginine through the activity of the nitric oxide synthases (NOS), of which there are 3 different forms, endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Bruckdorfer, 2005). Nitric oxide production via NOS requires molecular oxygen (Kwon *et al.*, 1990). The effects of NO are mediated by the second messenger cyclic guanine monophosphate (cGMP). The effects of NO on bone appear to be biphasic: slow release of NO leads to an anabolic effect on osteoblasts, leading to increased proliferation and alkaline phosphatase activity, whereas fast release and high levels of NO lead to inhibition of osteoblast proliferation and induction of apoptosis (Mancini *et al.*, 2000). These apparently contradictory effects appear to be controlled by the different NOS isoforms, with eNOS being responsible for the slow controlled release and anabolic responses, and iNOS being responsible for rapid high-level release and catabolic responses (Mancini *et al.*, 2000). The effects of oestrogen are mediated by NO signalling, as oestrogen has no protective effect on the bones of mice lacking eNOS (Armour *et al.*, 2001). Evidence is also accumulating that NO has an important role in exercise induced bone formation. Loading induced fluid shear stress stimulated the

synthesis of NO by osteocytes and osteoblasts and resulted in enhanced osteoblastic bone formation (Kapur *et al.*, 2003; Watanuki *et al.*, 2002; Zaman *et al.*, 1999).

### ***Nucleotides***

Nucleotides such as ATP and UTP are released by osteoblasts in response to a wide range of stimuli and are potent signalling molecules. They are detected through P2 receptors on osteoblasts and osteoclasts and ligand binding leads to intracellular calcium signalling (Hoebertz *et al.*, 2003). ATP and ADP increase the formation of osteoclasts and stimulate resorption by mature osteoclasts (Hoebertz *et al.*, 2001). ATP and UTP have strong inhibitory actions on bone formation by osteoblasts (Hoebertz *et al.*, 2002). ATP is released by endothelial cells in response to hypoxia, where it acts in an autocrine manner to induce release of vasodilators (Burnstock, 1987).

### ***Reactive oxygen species (ROS)***

ROS are produced enzymatically or as side-products of reactions utilising oxygen as an electron donor. RANKL stimulation leads to the production of ROS in osteoclasts and blockade of this ROS production inhibits osteoclastogenesis (Lee *et al.*, 2005). ROS have also been shown to stimulate osteoclastic resorption (Garrett *et al.*, 1990); the major species involved is hydrogen peroxide (Fraser *et al.*, 1996). As mentioned previously, production of ROS by TRAP has been implicated in the final stages of collagen breakdown from the resorption lacunae.

### ***Glucocorticoids***

Glucocorticoids (GC) are used to treat a variety of diseases including asthma and are potent anti-inflammatory agents. GC use has long been known to induce osteoporosis due to an inhibitory action on intestinal calcium absorption and increased renal calcium excretion (Lafage-Proust *et al.*, 2003). Whether they have direct effects on osteoclasts is hotly debated, however their inhibitory effects on osteoblasts are well documented. Osteoblast differentiation is stimulated by physiological concentrations of GC, however pharmacological concentrations inhibit collagen production and IGF production and *in vivo* markers of bone formation and

histomorphometry of bones show drastically reduced bone formation (Lafage-Proust *et al.*, 2003). Osteoblastic adhesion to extracellular matrix is decreased and matrix degradation is promoted by increased osteoblast expression of collagenases. GC increase circulating levels of endothelin-1, a potent vasoconstrictor, and this has been linked to glucocorticoids-induced osteoporosis and necrosis of the femoral head (Lafage-Proust *et al.*, 2003).

### ***Protons ( $H^+$ )***

The local environment plays a large role in cell activity, and pH is an important aspect of that environment. For every multicellular organism the problem arises of removal of waste from the local vicinity around cells. Protons and  $CO_2$  are produced as a consequence of cellular metabolism and excreted into the extracellular space; these must be removed from the cellular environment to prevent detrimental pH changes. The method utilised by the body for removal of these products is the vasculature. The bone acts as a reserve of base that acts to buffer blood pH changes by the release of  $HCO_3^-$  (Arnett, 2003). The  $HCO_3^- / CO_2$  buffering system controls the blood pH levels,  $CO_2$  release through expiration is central to this control. Protons are excreted via the kidneys and  $HCO_3^-$  is reabsorbed at this site. Alterations in  $CO_2$  expiration or kidney-regulated acid excretion lead to systemic acidosis. Disruption to the vasculature, for example at sites of infection, inflammation and in tumours, causes local acidosis by preventing the removal of waste  $CO_2$ , and limiting the provision of  $O_2$  leading to increased anaerobic metabolism and lactic acid production.

Increasing evidence indicates that extracellular protons function as signalling agents; several receptors have been identified on bone cells that are capable of detecting the presence of increased  $H^+$ . These include acid sensitive ion channels (ASICs) (Jahr *et al.*, 2005) and several G-protein coupled receptors (OGR1 and GPR4) (Ludwig *et al.*, 2003). Decreasing the pH to 6.9 in cultures of mature osteoclasts causes a dramatic stimulation of resorption to occur (Arnett *et al.*, 1994; Arnett and Dempster, 1986; Arnett and Spowage, 1996); however, continuous culture at low pH in long-term cultures leads to decreased osteoclast formation. The stimulatory effect of acidosis on osteoclast activity is unusual, because acidosis is normally detrimental to cell activity. This is certainly true of the effect of acidosis on

osteoblasts; low pH has strong inhibitory effects on osteoblast mineralisation (Brandao-Burch *et al.*, 2005).

## **Tissue oxygenation**

In mammals, O<sub>2</sub> is acquired through inhalation of atmospheric air, which contains approximately 21% O<sub>2</sub>, into the lungs, where O<sub>2</sub> enters the arterial circulation through gas exchange across the alveolar wall. At the same point, CO<sub>2</sub> waste from cell respiration diffuses from the blood into the lungs and is exhaled. O<sub>2</sub> is relatively insoluble in blood, and is carried in solution bound to the protein haemoglobin in red blood cells. Haemoglobin binds oxygen cooperatively; the binding of the first molecule lowers binding energy for further molecules, up to a maximum of 4 O<sub>2</sub> molecules per haemoglobin. O<sub>2</sub> binding to haemoglobin requires coordination with a Fe<sup>2+</sup> ion; hence anaemia leads to lower levels of blood oxygenation due to decreased O<sub>2</sub> binding to haemoglobin. The affinity of O<sub>2</sub> for haemoglobin is decreased by increasing temperature, pCO<sub>2</sub>, or protons; this effect is vital for increasing oxygen supply to active muscles which are hot, hypercarbic and acidic.

O<sub>2</sub> dissociates from haemoglobin and diffuses down a gradient from capillaries to the individual cells, where the mitochondria act as O<sub>2</sub> sinks. Virtually all cells need to be within 100 µm of a capillary to ensure adequate oxygenation (Bertolini *et al.*, 2000). CO<sub>2</sub> diffuses from where it is produced within cells to capillaries and venules to be transported to the lungs and released upon exhalation. Arterial blood contains approximately 12% O<sub>2</sub> and venous blood has approximately 5% O<sub>2</sub>, which leads to a range of between 5 and 12% O<sub>2</sub> in normal tissues. The amount of O<sub>2</sub> delivered to a tissue depends on a number of factors; the amount of oxygen freely dissolved in arterial blood (standard at 0.003 ml O<sub>2</sub> / 100ml), the amount of oxygen carried by haemoglobin (1g of haemoglobin can carry ~1.39 ml O<sub>2</sub> at 100% saturation, normal blood has ~15g of haemoglobin / 100ml, therefore ~20.8 ml O<sub>2</sub> / 100ml), and the rate of blood flow (cardiac output and vascular density).

## ***Oxygen sensing***

All organisms from bacteria to humans possess mechanisms to maintain O<sub>2</sub> homeostasis (Semenza, 1999). The importance of oxygen in the process of oxidative phosphorylation means that organisms using this as their primary source of energy need to ensure that oxygen supply is sufficient for their energy requirements. Hypoxia can result in a failure to generate sufficient ATP to maintain cellular function, whereas hyperoxia generates reactive oxygen intermediates that can be harmful to membranes and DNA. Hence, cellular O<sub>2</sub> levels must be regulated within tight physiological limits.

Several pathological situations are commonly associated with hypoxia. Infection and inflammation lead to disruption of the blood supply and subsequent localised hypoxia (Lewis *et al.*, 1999). Solid tumours are often highly vascularised; however, the vessels are generally disorganised and 'leaky' due to inadequate pericyte support, leading to hypoxia within the tumour (Vaupel *et al.*, 1989). Bone fracture leads to interruption of the blood supply and hypoxia at the fracture site (Brighton and Krebs, 1972). Systemic hypoxia can occur in patients with chronic obstructive pulmonary disease, due to decreased inspired O<sub>2</sub>, and congestive heart failure, due to decreased perfusion (Peers and Kemp, 2004). Perfusion also generally decreases during ageing, due to decreased elasticity of vessels and reduced cardiac output. Arterial oxygen tension (pAO<sub>2</sub>) is generally maintained, but alterations in the vessel responses to vaso-constrictors and -dilators mean that vessels are less able to respond quickly to changes in oxygenation, leading to more frequent episodes of local hypoxia (Janssens *et al.*, 1999).

Hypoxia can occur either locally or systemically, and different mechanisms are utilised for detecting these oxygen changes. Systemic hypoxia requires a rapid, whole organism response in order to ensure that the essential organs receive sufficient oxygenation to keep the organism alive. Localised hypoxia, usually caused by vessel occlusion, only affects a small area of cells, and the response is tailored towards reinstatement of the blood supply and cell survival. The systems used to detect and respond to these hypoxic situations are described in detail below.



## ***Systemic oxygen sensing***

Specialised innervated regions such as the carotid and aortic bodies, and neuroepithelial body in the lung are capable of sensing  $pO_2$  in the blood and inspired air respectively (Peers and Kemp, 2004). Cells within these regions are specialised chemoreceptors; responsiveness to hypoxia is conferred by the presence of  $K^+$  channels on the cell membrane (Peers and Kemp, 2004). Several different types of oxygen sensitive  $K^+$  channels have been identified: voltage-gated  $K^+$  channels ( $K_v$ ),  $Ca^{2+}$ -activated  $K^+$  channels ( $K_{Ca}$ ), and TASK-like background  $K^+$  channels. All hypoxia-sensitive neurosecretory cells identified to date express these ion channels (Lopez-Barneo *et al.*, 2004). Decreased  $pO_2$  closes  $K^+$  channels, leading to membrane depolarisation and/or increase of action potential firing frequency, influx of extracellular  $Ca^{2+}$  through voltage gated channels and subsequent transmitter release to the extracellular space (Lopez-Barneo *et al.*, 2004). These signals regulate acute responses to oxygen deficiency, including hyperventilatory responses and increased heart output. Hypoxia also causes vasodilation in coronary and carotid vessels, which provide blood to the heart and brain, ensuring oxygenation to these essential organs, this response is mediated through ATP sensitive  $K^+$  channels ( $K_{ATP}$ ), and is triggered by decreased ATP production during hypoxia (Lopez-Barneo *et al.*, 2004).

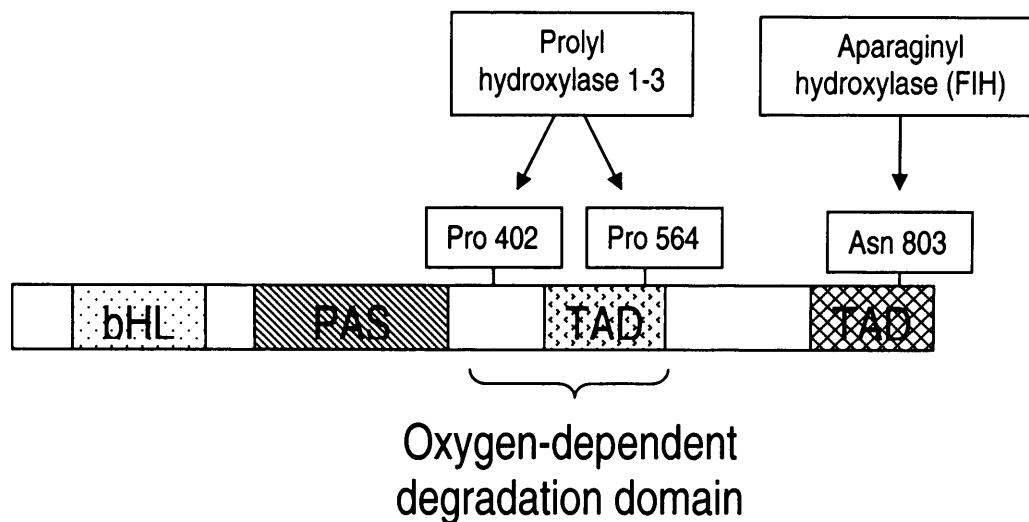
Ion channels provide a rapid method to respond to acute periods of systemic hypoxia, with responses centred around maintenance of essential functions and increasing oxygen supply on a whole organism level. The cells that contain oxygen sensitive  $K^+$  channels are responsible for systemic oxygen regulation, however every cell within the body requires oxygen for energy production and needs to be able to respond individually to localised chronic hypoxia. At the cellular level, a highly conserved and universal signalling pathway exists, which transmits  $O_2$  information to the nucleus where transcription is altered to mediate  $O_2$  dependent gene expression, leading to enzymes that allow the cell to produce enough energy for survival until hypoxia is relieved. The elucidation of the mechanism by which this information is transferred to the nucleus is still occurring.

## ***Hypoxia inducible factor***

The hypoxia inducible factor was first identified after studies on transcriptional control of the erythropoietin gene. It was known that hypoxia stimulated erythropoietin gene expression, and through investigation of the transcription factors binding to the promoter region of this gene, the hypoxia inducible factor (HIF) was identified (Fandrey, 1995). HIF consists of 2 subunits, both of which exist in multiple forms, with molecular masses of 120-130 kDa and 91-94 kDa and designated HIF $\alpha$  and HIF $\beta$  respectively (also known as the Aryl hydrocarbon Receptor Nuclear Translocator, or ARNT) (Semenza, 1999). The HIF subunits contain basic helix loop helix (bHLH) and Per Arnt Sim (PAS) domains (**Figure 1.4**), which control DNA binding and dimerisation (Semenza, 1999). Expression of the HIF $\alpha$  and HIF $\beta$  subunits is constitutive; however, HIF $\alpha$  protein is rapidly degraded in normoxia.

The mechanism by which HIF $\alpha$  degradation occurs has only recently been elucidated. Investigations into the hereditary cancer syndrome caused by germ line mutations in the Von Hippel Lindau (VHL) gene, associated with highly vascularised tumours, found transcription of hypoxia-associated genes was up-regulated (Iliopoulos *et al.*, 1996). This led to the discovery that VHL protein (pVHL) interacts with the HIF $\alpha$  subunit; pVHL recognises HIF $\alpha$  via 2 hydroxyproline residues in the oxygen-dependent degradation domain of the HIF $\alpha$  protein. Binding of pVHL to HIF $\alpha$  leads to polyubiquitination of the HIF $\alpha$  subunit and its subsequent degradation (Cockman *et al.*, 2000; Maxwell *et al.*, 1999; Tanimoto *et al.*, 2000). This provides a mechanism by which oxygen directly modulates gene expression, since prolyl hydroxylases require molecular oxygen. Three conserved prolyl-4-hydroxylases have been identified which interact directly with HIF $\alpha$ . These are designated as prolyl hydroxylase domain containing 1, 2 and 3 (PHD1-3) (Epstein *et al.*, 2001); their activity is regulated directly by oxygen tension. PHD1 is expressed constitutively, whereas PHD2 and PHD3 are induced by hypoxia (Metzen *et al.*, 2003).

HIF $\alpha$  is not only regulated by oxygen at the level of protein stability. Further hydroxylation occurs at an asparagine residue in the C-terminal transactivating domain (C-TAD) that disrupts HIF binding to its co-activator c300/CBP, leading to



**Figure 1.4: Structure of the hypoxia inducible factor  $\alpha$ .**

HIF $\alpha$  consists of basic helix loop helix domain (bHLH) and a Per Arnt Sim (PAS) domain which regulate DNA binding and dimerisation with HIF $\beta$  respectively. N-terminal and C-terminal transactivation domains (TAD) govern interactions with transcriptional coactivators. Proline residues at aa 402 and 564 are hydroxylated by the HIF prolyl hydroxylases in the presence of molecular oxygen within a region termed the oxygen dependent degradation domain, this regulates recognition by the Von-Hippel Lindau tumour suppressor protein and proteasomal destruction of HIF $\alpha$  in the presence of oxygen. An asparagine residue at aa 803 is hydroxylated by Factor Inhibiting HIF (FIH) which prevents the interaction between HIF and its transcriptional coactivator Creb Binding Protein (CBP) / p300.

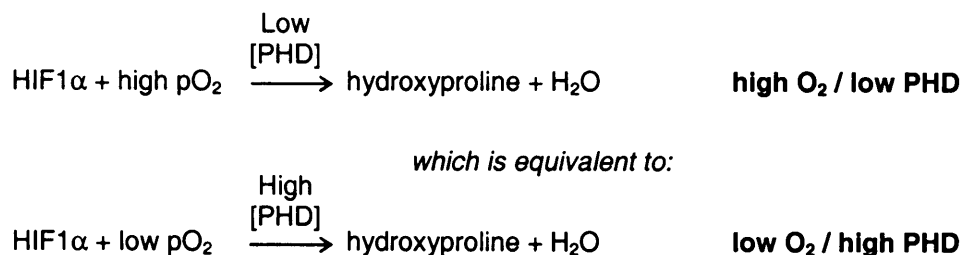
repression of transcriptional activity (Lando *et al.*, 2002). This hydroxylation is performed by factor inhibiting HIF (FIH), an asparaginyl hydroxylase. Hence, oxygen is capable of regulating gene expression by acting as an essential substrate for enzymes that hydroxylate specific proline and asparagine residues within the HIF $\alpha$  peptide, leading to degradation and loss of activity in normoxia.

The initial identification of the HIF PHDs occurred in the nematode worm *C. elegans*, indicating that the HIF signalling system is highly conserved (Epstein *et al.*, 2001). The downstream effects of HIF activation are transcription of specific genes associated with cell activity and survival. In general, genes activated by HIF function to maintain cell energy supplies during hypoxia, e.g. glycolytic enzymes and glucose transporters, and to provide increased O<sub>2</sub> delivery; examples include erythropoietin and vascular endothelial growth factor (VEGF). The effect of hypoxia on specific cell types is discussed in detail below.

Several HIF $\alpha$  subunits have been described, with HIF1 $\alpha$  being the most studied to date. HIF1 $\alpha$  is expressed ubiquitously throughout the body and is indispensable for survival. HIF1 $\alpha$  knockout mice show abnormal vascular development, failure of neural tube closure, and extensive mesenchymal cell death leading to death by embryonic day 11 (Iyer *et al.*, 1998; Kotch *et al.*, 1999; Ryan *et al.*, 1998). Further investigation in HIF1 $\alpha$  deficient cell lines revealed that the glycolytic response to hypoxia appears to be regulated exclusively by HIF1 $\alpha$  (Hu *et al.*, 2003). HIF2 $\alpha$  shares 48% overall amino acid identity with HIF1 $\alpha$  (Bracken *et al.*, 2005); however, in the bHLH (DNA binding) and PAS (HIF $\beta$  binding) domains that homology increases to 83% and 70% respectively (Hu *et al.*, 2003). The critical proline residues in the oxygen-dependent degradation domain are also conserved, as are the terminal 50 amino acids that regulate p300 binding and transcriptional co-activation (Hu *et al.*, 2003). HIF2 $\alpha$  knockout animals show variable phenotypes, with some offspring surviving to term (Tian *et al.*, 1998). Those animals surviving to term have multiple organ pathologies including retinopathy, hepatic steatosis, cardiac hypertrophy, and mitochondrial dysfunction as well as severe abnormalities in haematopoiesis (Scortegagna *et al.*, 2003a; Scortegagna *et al.*, 2003b). These studies indicate that the functions of HIF1 $\alpha$  and HIF2 $\alpha$  are non-redundant and do not overlap. A third

variant, HIF3 $\alpha$ , has also been described; it is induced by hypoxia and is capable of binding to HIF $\beta$ , but lacks the N-terminal transactivation domain (Hara *et al.*, 2001). Its role in hypoxia-induced signalling seems to be as a negative regulator of HIF $\alpha$  activity (Makino *et al.*, 2002).

The mechanism by which the oxygen tension ‘set point’ to which cells respond is established appears to be through differential expression of the three HIF prolyl hydroxylases. Proline hydroxylation is a non-equilibrium process and increasing or decreasing the amount of enzyme will alter the reaction kinetics and ‘set’ the oxygen tension at which HIF is stabilised; the higher the PHD concentration, the lower pO<sub>2</sub> threshold for HIF activation (Maxwell and Salnikow, 2004). For example:



Decreasing the amount of O<sub>2</sub> whilst holding PHD steady leads to decreased hydroxyproline formation and stabilisation of HIF $\alpha$ . The mechanism by which cells adapt to hypoxia is via altering the expression of PHDs. The PHDs are hypoxia inducible and this acts to reset the cells O<sub>2</sub> threshold, preventing excessive, continual HIF signalling.

There are indications that the formation of reactive oxygen species (ROS) within the mitochondria might mediate hypoxic signalling (Waypa *et al.*, 2001). This is difficult to analyse due to the transient nature of ROS, and lack of agreement as to whether ROS production increases or decreases during hypoxia. O<sub>2</sub> serves as an electron acceptor in the electron transport chain (ETC) in mitochondria. It is ultimately reduced to H<sub>2</sub>O by the addition of two electrons. The incomplete reduction of O<sub>2</sub> to H<sub>2</sub>O leads to the production of superoxide, which can be converted to H<sub>2</sub>O<sub>2</sub> spontaneously, or by the enzyme superoxide dismutase. Superoxide is produced at low levels by the ETC during normal metabolism. The disagreement arises as to

whether decreasing the supply of O<sub>2</sub> will lead to incomplete reduction of O<sub>2</sub> by the ETC, resulting in increased superoxide production, or whether it will inhibit activity of the ETC generally, resulting in less superoxide production. Evidence has been presented for both theories (for review see Kietzmann and Gorlach, 2005).

There is strong evidence that phosphorylation of p38 MAPK is required for stabilisation of HIF. p38 kinase <sup>-/-</sup> cells are unable to stabilise HIF in response to hypoxia and disruption of the upstream kinase pathway, MKK3 <sup>-/-</sup> and MKK6 <sup>-/-</sup> cells, causes the same defect, indicating that p38 is essential for the stabilisation and transcriptional activity of HIF (Emerling *et al.*, 2005). This p38 activity appears to be regulated via the production of hydrogen peroxide through complex III of the mitochondrial respiratory chain (Emerling *et al.*, 2005).

HIF is not only activated by hypoxia. Growth factors, vascular hormones, and cytokines can increase HIF activity by augmenting transcription of HIF1 $\alpha$  message as well as stabilisation of HIF $\alpha$  (Dery *et al.*, 2005; Treins *et al.*, 2005). It has also been suggested that the hydroxylases that modify HIF $\alpha$  are not the primary oxygen sensors. It has also been demonstrated that HIF $\alpha$  stabilisation is lowered in situations of hyperosmolarity such as in diabetes leading to deficient hypoxia detection (Catrina *et al.*, 2004).

Nitric oxide (NO) and its intermediates are also capable of stabilising HIF $\alpha$  in the absence of a hypoxic stimulus. However, in the presence of hypoxia, the opposite occurs: HIF $\alpha$  degradation is induced by NO (Callapina *et al.*, 2005). The reasons for this reversal are unclear but may be due to interactions between NO and the HIF prolyl hydroxylases (Callapina *et al.*, 2005). Pyruvate and oxaloacetate, intermediates in glycolytic metabolism, stabilise HIF $\alpha$  in normoxia, and this effect is regulated independently to hypoxic induction (Lu *et al.*, 2005). They reversibly inhibit the activity of the HIF PHDs by binding to the active site in preference to 2-oxoglutarate; this method of HIF stabilisation is inhibited by the amino acids histidine and cysteine as well as by ascorbate (Lu *et al.*, 2005). The supply of oxygen is essential for efficient glucose metabolism, the build up of metabolic intermediates would serve as an indicator of inefficient processing and act to increase energy metabolism via anaerobic glycolysis.

Although the HIF system is the main pathway for transmitting oxygen information to the nucleus, other intracellular signalling pathways have also been identified as responding to changes in  $pO_2$ . These pathways may be responding directly to  $pO_2$ , or they may detect changes in redox status in the cell induced by lack of  $O_2$ . Oxygen-sensitive transcription factors include NF $\kappa$ B, which is involved in most stress responses and is activated by hyperoxia; this activation can be inhibited by the addition of antioxidants, indicating that reactive oxygen species are critical to the oxygen dependent activation (Haddad, 2002). Hypoxia also induces a rapid influx of calcium from the extracellular space that could directly activate calcium sensitive pathways (Seta *et al.*, 2004). Furthermore, the change to anaerobic glycolysis and decreased perfusion commonly associated with hypoxia will lead to a concomitant drop in pH, which could mediate some of the observed effects of hypoxia.

## **Bone and the vasculature**

Bone is a highly vascular tissue, reflecting the requirement for nutrients and diffusible ions. The process of haematopoiesis also requires an adequate circulatory system in the marrow to allow the exit of newly formed cells into the circulation and to allow the signals governing their development to be transduced from peripheral sites to the marrow.

Blood is supplied to the bone via nutrient arteries that enter the marrow space through shafts traversing the cortical bone. There are generally 1 or 2 principal arteries that perforate the diaphysis to irrigate the marrow space, and many smaller vessels that supply the epiphysis and metaphysis (Brookes and Revell, 1998). Early in life the blood supply is centrifugal; blood flows from the marrow space through the cortical bone to efferent veins and sinusoids at the periosteal surface (**Figure 1.5**).

As individuals age, the blood supply becomes more dependent on periosteal arteries to supply the cortical bone as the marrow supply diminishes due to atherosclerosis of the nutrient arteries (Bridgeman and Brookes, 1996). It has been demonstrated that ageing bone marrow is ischaemic (Brookes and Revell, 1998). Other pathological situations in bone associated with vascular disruption and reduced oxygen tension are discussed below.



**Figure 1.5: Vascular channels into the cortical bone of rat femur.**

SEM image indicating the density of vascular channels penetrating the inner surface of cortical bone from the marrow space.

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### ***Fracture healing***

During fracture healing the events observed during intramembranous and endochondral ossification are recapitulated. The initial stage of fracture healing is the formation of a haematoma and inflammation around the fracture site. Following this initial inflammatory response, a callus forms around the fracture site; the external bony surface of this callus is formed via intramembranous ossification by osteoprogenitor cells in the periosteum. The internal regions of the callus are composed of cartilage, which is also formed by cells derived from the periosteum (Einhorn, 1998). The cartilage is invaded by blood vessels and subsequently becomes mineralised, followed by extensive removal of the mineralised cartilage and replacement with bone as is observed during endochondral ossification (Einhorn, 1998). Sites of bone fracture are hypoxic (Brighton and Krebs, 1972) and depend upon the successful re-establishment of the blood supply for healing (Street *et al.*, 2002).



## ***Osteonecrosis***

Osteonecrosis occurs when damage to the bone leads to cell death and the bone is degraded and removed. Osteonecrosis can occur due to glucocorticoids use, which decreases blood flow to the bone by increasing endothelin-1 levels (Lafage-Proust *et al.*, 2003). Osteonecrosis of the femoral head is preceded by bone marrow ischaemia, and ligation of the arteries supplying the femoral head in animals leads to osteonecrosis (Laroche, 2002). The death of the bone is linked to the death of osteocytes within the bone matrix that maintain the viability of the tissue; loss of the blood supply causes ischaemia and osteocyte death (Winet *et al.*, 1998).

## ***Rheumatoid arthritis (RA) and osteoarthritis (OA)***

The pathological changes associated with rheumatoid arthritis begin with an inappropriate immune response, leading to inflammation in the joint, and pannus formation. Synovial fibrocytes and T lymphocytes release destructive enzymes and cytokines that enhance the matrix destruction and inflammation leading to a perpetuating inflammatory response and a feedback loop (Firestein, 2003). One of the notable features of RA is the highly vascularised pannus, VEGF is found in the synovial fluid of RA patients and its levels are correlated with disease severity (Clavel *et al.*, 2003). Furthermore, reduction of VEGF by addition of synthetic or natural inhibitors leads to lessening of symptoms (Giatromanolaki *et al.*, 2003). One of the strongest impetuses for VEGF production is hypoxia, and RA joint fluids have lower pO<sub>2</sub> than normal synovial fluid despite increased vascularisation (Lund-Olesen, 1970; Taylor and Sivakumar, 2005). The reason for this is that the vasculature induced by the disease is mainly formed of disorganised capillaries, which are unable to regulate blood flow and oxygen delivery adequately, and the high cellularity of RA tissue leads to hypoxia (Taylor and Sivakumar, 2005). HIF1 and 2 $\alpha$  are both upregulated in synovial fibrocytes, and are associated with increased angiogenesis and VEGF production in osteoarthritis (OA) (Giatromanolaki *et al.*, 2003).

Many of the inflammatory mediators released into the pannus during RA are hypoxia-inducible and angiogenic, in particular, VEGF, TNF $\alpha$ , and IL-1 (Clavel *et al.*, 2003). TNF $\alpha$  and IL-1 have also been shown to stabilise HIF proteins in

normoxia (Hellwig-Burgel *et al.*, 2005). It should be noted that these factors are also osteoclastogenic, and this may help explain the joint destruction and bone loss associated with RA.

### ***Paracrine / endocrine interactions between bone and the vasculature***

In addition to its controlling action on osteoclastogenesis, RANKL also exhibits angiogenic activity. RANKL is produced by vascular smooth muscle cells and can be detected by RANK on the surface of endothelial cells, where ligand binding results in prevention of apoptosis (Kim *et al.*, 2003). Expression of RANK on endothelial cells is upregulated by VEGF and this subsequently enhances the angiogenic response to RANKL (Min *et al.*, 2003). Expression of RANKL has also been demonstrated in bone-derived vascular endothelial cells and RANKL is up regulated in response to TGF $\beta$ , TNF $\alpha$ , and IL-1 $\alpha$  (Collin-Osdoby *et al.*, 2001; Ishida *et al.*, 2002). These interactions between the vascular system and bone have been hypothesised to play a role in atherosclerotic changes in the vascular endothelium (Collin-Osdoby, 2004). These findings also suggest a mechanism by which the activity of the basic multicellular unit can be regulated; the production of RANKL by osteoblasts acting upon both osteoclasts and endothelial cells.

The link between cardiovascular disease and increased risk for osteoporosis is well established. Interestingly, bone mineral density is a better predictor for stroke and heart attack than blood pressure (Browner *et al.*, 1991). It has been suggested that the link between osteoporosis and vascular disease is due to shared risk factors such as ageing; however, partial occlusion of the blood supply to one limb whilst the other is unaffected leads to reduced bone mineral density only in the occluded limb (Laroche, 2002).

The skeletal and vascular systems both experience detrimental changes in postmenopausal women. Oestrogen induces the production of eNOS, which in turn increases levels of NO in the vascular endothelium. NO is a vasodilator and protects against atherosclerosis, decreased oestrogen following the menopause leads to lower levels of NO and subsequent increased risk of atherosclerosis (Chambliss and Shaul, 2002). NO production through eNOS has direct effects on osteoblasts (Mancini *et*

*al.*, 2000); loss of oestrogen stimulated eNOS production in the bone vasculature may exacerbate the bone loss associated with oestrogen withdrawal following the menopause (Armour *et al.*, 2001). Interestingly, both NO and oestrogen require molecular oxygen for their synthesis (Estabrook, 2005; Kwon *et al.*, 1990).

Statins are a class of drug that have been used to improve cardiovascular health but have subsequently been suggested to have anabolic effects on the skeleton. This interaction has prompted the investigation into the possible mediators of this shared protective effect. Statins function by inhibiting the mevalonate pathway, this leads to downstream effects such as increased eNOS synthesis and activity as well as reductions in serum cholesterol (Whitney *et al.*, 2004). The reduction in cholesterol leads to decreased risk of coronary heart disease, the increased production of NO induced by statin usage is also likely to have a major role in the vascular protective effect (Whitney *et al.*, 2004). Statins function upstream of bisphosphonates in the mevalonate pathway and as such it is not surprising that they have a positive effect on the skeleton. However rather than simply suppressing bone turnover as bisphosphonates do (Reszka and Rodan, 2003), statins actually increase bone formation through increased production of BMP2 in osteoblasts (Mundy *et al.*, 1999), it is possible that the effects of statins on the vasculature could also be having beneficial effects on bone.

## **Effects of hypoxia on cells of bone and related lineages**

### *Haematopoietic cells*

Haematopoietic cell development occurs in the bone marrow in distinct regions located around sinuses in the blood vessels. These vessels allow the egress of mature haematopoietic lineage cells into the circulation. The initial stages of stem cell maturation occur in the extremities of the bone marrow, adjacent to the bone surface. Modelling studies of the bone marrow circulation and oxygen distribution indicate that oxygen tension at the bone surface would be significantly lower than that experienced by the more mature cells near the vascular sinus (Chow *et al.*, 2001). Haematopoietic and committed progenitor cell proliferation and differentiation vary depending on pO<sub>2</sub> and this is correlated to the location of the different lineages within

the bone marrow compartment. Several studies have documented the response of immature haematopoietic cells to decreased oxygen. Culture in hypoxia (1% O<sub>2</sub>) maintains the marrow repopulating capacity of isolated haematopoietic stem cells (HSCs), and, when cultures are supplemented with various cytokines, also leads to the proliferation of more mature, lineage committed progenitors (Bradley *et al.*, 1978; Danet *et al.*, 2003; Ivanovic *et al.*, 2000; Ivanovic *et al.*, 2002; Ivanovic *et al.*, 2004). Within the architecture of the bone marrow, megakaryocyte and erythrocyte maturation occurs in clusters closer to the vessel sinus than granulocyte maturation (Tavassoli and Yoffey, 1983). This correlates with the increased pO<sub>2</sub> at which megakaryocyte and erythrocyte maturation occurs (enhanced in 20% O<sub>2</sub>) (LaIuppa *et al.*, 1998) compared to that of granulocytes (enhanced in 5% O<sub>2</sub>) (Hevehan *et al.*, 2000).

The effects of hypoxia on mature circulating and tissue resident haematopoietic cells have also been investigated. A common consequence of infection and injury is the creation of a hypoxic environment around the site. 95% of neutrophils and macrophages present at these sites are recruited there from the circulation rather than initially resident in the tissue (Lewis *et al.*, 1999). Therefore, these cells need to be able to travel against the oxygen gradient to the site and once there, generate sufficient energy to function within this low oxygen environment.

### *Macrophages*

Macrophages play an essential role in innate immunity and are close relatives of osteoclasts. Hypoxia increases the expression and production of TNF $\alpha$  and VEGF, and enhances NO production by macrophages (Lewis *et al.*, 1999). Differentiation of bone marrow macrophages in low pO<sub>2</sub> (<5%) led to the formation of cells that were more effective phagocytes and antigen presenting cells and more responsive to lipopolysaccharide (LPS) stimulation than counterparts formed in normoxia (20% O<sub>2</sub>) (Pfau *et al.*, 2004). The phenotype of normoxic macrophages was more consistent with that of alveolar macrophages, whereas those formed in low oxygen had characteristics more typical of peritoneal macrophages, indicating the physiological relevance of differing oxygen tensions on macrophage function (Pfau *et al.*, 2004). Hypoxia also decreases the expression of macrophage chemoattractant protein (MCP)

by macrophages and fibroblasts (Bosco *et al.*, 2004). This could lead to the localisation of macrophages to sites of tumour, infection and inflammation due to a lack of chemotactic stimulus (Lewis *et al.*, 1999). The macrophage inflammatory response is mediated by, and dependent upon, the hypoxia inducible factor (Cramer *et al.*, 2003). Macrophages derive a large proportion of their energy from glycolysis, mitochondrial inhibitors have little or no effect on macrophage chemotaxis, aggregation or invasion (Peyssonau and Johnson, 2004); this allows them to function in low oxygen environments. Expression of glycolytic genes is controlled by HIF and deletion of HIF1 $\alpha$  from peritoneal macrophages led to large decreases in cellular ATP pools, even when cells were cultured in normoxia (Cramer *et al.*, 2003). HIF1 $\alpha$ <sup>-/-</sup> macrophages, derived by myeloid cell-specific knockdown of HIF1 $\alpha$ , displayed significantly impaired chemotaxis, bacterial killing and invasion (Cramer *et al.*, 2003). The effects of hypoxia on macrophages may not always be beneficial. Macrophages are commonly found within hypoxic sites in tumours, where they may be major contributors to tumour angiogenesis because as they produce large quantities of VEGF and TNF $\alpha$  due to HIF2 $\alpha$  stabilisation; this is associated with poor outcome (Leek *et al.*, 2002).

### *Neutrophils*

Neutrophils are circulating cells that play an essential role in innate immunity. Hypoxia inhibits the apoptosis of neutrophils and this effect is regulated by HIF $\alpha$  dependent NF $\kappa$ B activation (Walmsley *et al.*, 2005). As demonstrated for macrophages, loss of HIF1 $\alpha$  in neutrophils also leads to depletion of intracellular ATP stores and cell death (Cramer *et al.*, 2003). Apoptosis and removal of neutrophils is essential for the cessation of inflammatory responses.

### *Mesenchymal cells*

#### *Chondrocytes*

Chondrocytes are responsible for the production of cartilage, a connective tissue comprised mainly of type II collagen. Cartilage lines the joint surfaces and is required to protect the joint from damage due to load bearing. Due to the

compressive forces it experiences cartilage is avascular; chondrocytes embedded within the cartilaginous matrix rely on the synovium for their nutrients. The synovial fluid has an oxygen tension of approximately 7%, models of oxygen consumption by chondrocytes indicate that cells within the deeper layers of cartilage are experiencing oxygen tensions around 1% (Grimshaw and Mason, 2000; Zhou *et al.*, 2004). Chondrocytes are adapted to low oxygen tension and derive as much as 75% of their energy from anaerobic glycolysis, and the chondrocyte phenotype is maintained *in vitro* by exposure to reduced oxygen tension (Rajpurohit *et al.*, 1996). Furthermore, culture of chondrocytes in high pO<sub>2</sub> leads to dedifferentiation, transfer into low pO<sub>2</sub> (2% or 5%) results in redifferentiation of the chondrocytic phenotype (Domm *et al.*, 2002). Exposure of bovine articular chondrocytes to oxygen tensions ranging from 0.1% to 20% revealed that chondrocytes are capable of surviving severe hypoxia for extended periods of time, and also that pO<sub>2</sub> below 5% led to reduced matrix production (Grimshaw and Mason, 2000).

The chondrocytic survival response to low oxygen tension is dependent upon the presence of HIF1 $\alpha$ , as chondrocytes in the depths of the growth plate of cartilage specific HIF1 $\alpha$  knockout animals die (Schipani *et al.*, 2001). Normal human articular chondrocytes consistently stabilise HIF1 $\alpha$  in normoxia and increase its level in hypoxia, so it appears that HIF1 $\alpha$  may play a role in normal chondrocyte function (Coimbra *et al.*, 2004). HIF2 $\alpha$  also plays a role in chondrocyte biology as HIF2 $\alpha$ <sup>-/-</sup> mice have abnormal growth plate development (Scortegagna *et al.*, 2003), and chondrocytes in hypertrophic cartilage increase production of this factor as they progress through their development (Stewart *et al.*, 2006).

### *Fibroblasts*

When connective tissue is damaged during a wounding process the local blood supply is disrupted and the wound healing process must begin in a situation of decreased blood supply and hypoxia. Where the process of bone fracture healing is one of regeneration of damaged tissue, wound healing is more of a reparative process. Scarring is the outcome of fibroblastic activity to heal the wounded tissue. It has been established that scarring is due to the fibroblastic production of collagenous matrix, controlled by transforming growth factor  $\beta_{1-3}$  (TGF $\beta_{1-3}$ ) (Mauviel, 2005).

Hypoxia at the wound site is capable of controlling TGF $\beta$  gene expression and has been implicated as a pro-fibrotic factor (Scheid *et al.*, 2002). Hypoxia has also been shown to inhibit the proliferation of normal human dermal fibroblasts (NHDF) (Oberringer *et al.*, 2005). The production of VEGF is dramatically increased in NHDF exposed to low pO<sub>2</sub>, as is the expression of collagen type 1 although this increase is only sustained for a very short period of time (24 hours), likely due to the requirement for oxygen for successful hydroxylation of the collagen molecules (Steinbrech *et al.*, 1999).

### *Adipocytes*

A recent report details the effects of hypoxia on adipocytes, observing decreased proliferation and adipogenesis (Kim *et al.*, 2005), confirming an earlier report identifying decreased PPAR $\gamma$  expression in response to hypoxia, a mechanism regulated by HIF1 $\alpha$  (Yun *et al.*, 2002). A previous study had found that lipid accumulation increased in bone marrow derived stromal cells, although this was not accompanied by increased expression of adipogenic factors, suggesting that the lipids were not associated with adipocytes (Fink *et al.*, 2004). This finding has relevance to the process of atherosclerosis, where lipid accumulations are found in foam cells and are a hallmark of the disease; hypoxia has been shown to increase lipid accumulation by macrophages (Lattimore *et al.*, 2005). A further effect of hypoxia on mature adipocytes is the increased production of the angiogenic and bone modulating hormone leptin (Grosfeld *et al.*, 2002). Hypoxia probably alters this hormone due to its effects on energy homeostasis.

### *Osteoblasts*

As for wound healing, the fracture of a bone disrupts the vasculature to the site and leads to a zone of hypoxia around the fracture (Brighton and Krebs, 1972). The success of fracture healing depends on the successful reestablishment of the blood supply (Brookes and Revell, 1998). The link between angiogenesis and bone formation is well established, both during development and during fracture healing. However, the significance of the oxygen tension has not been clearly determined, rather the functional regulators of angiogenesis, many of them potentially induced by

hypoxia, have received more attention. Amongst the studies investigating the effect of  $pO_2$  on bone a confusion of results abounds. Researchers have observed decreased proliferation and increased alkaline phosphatase expression (Steinbrech *et al.*, 1999), and increased proliferation and increased alkaline phosphatase activity (Lennon *et al.*, 2001), whilst others claim decreased alkaline phosphatase activity (Ontiveros *et al.*, 2004; Park *et al.*, 2002). These disagreements are likely due to the usage of transformed cell lines, which have altered gene expression and differentiation profiles to primary cells (Ontiveros *et al.*, 2004), and different interpretations of what constitutes hypoxia. General consensus is that *runx2* expression is decreased by hypoxia (Akeno *et al.*, 2001; Ontiveros *et al.*, 2004; Park *et al.*, 2002; Salim *et al.*, 2004), and VEGF production is increased by hypoxic osteoblasts (Akeno *et al.*, 2001; Akeno *et al.*, 2002; Kim *et al.*, 2002; Steinbrech *et al.*, 1999).

## **Thesis aims**

Hypoxia has striking effects on other cell types and pathological bone loss is often associated with decreases in vascular perfusion, for example at sites of infection, inflammation, fracture, and tumours. Furthermore, many cytokines that are osteoclastogenic are angiogenic, and efficient vascularisation is essential for the processes of endochondral ossification and fracture healing. A common thread linking these processes is the presence or absence of oxygen. Therefore, I have investigated the effects of hypoxia on osteoclast formation and mineralised tissue resorption in rat and human model systems. I also investigated the effects of hypoxia on the differentiation and mineralised matrix forming potential of osteoblasts derived from rats.



# CHAPTER 2

## *Hypoxia stimulates the formation but not activity of rodent osteoclasts*

### **Introduction**

Mature osteoclasts isolated from the long bones of rats and rabbits have been extensively used to study agents that can activate or inhibit bone resorption, due to their ease of isolation and relative purity. Isolated mature osteoclasts are capable of being activated to resorb bone or dentine, and express the osteoclast marker tartrate resistant acid phosphatase (TRAP) (Boyde *et al.*, 1984; Chambers *et al.*, 1984). The activation of mature osteoclasts is dependent upon acidification of the extracellular environment (Arnett and Dempster, 1986). When these cells are exposed to control and test situations, the level of osteoclast activity can be determined by counting the number, area or volume of the resorption pits formed by these cells (Arnett and Dempster, 1987). This assay can also give clear and direct indication of toxicity of the experimental treatment, due to increases or decreases in osteoclast numbers. A disadvantage of this method is that stromal cells are present in the preparations, which can affect the activity of osteoclasts in response to treatments.

Whilst the mature osteoclast model is useful for studying the effect of treatments on osteoclast activity, it is not suitable for investigating the formation of osteoclasts. The study of osteoclast formation requires the culture of myeloid lineage cells from bone marrow or spleen preparations in the presence of the osteoclast differentiation factors M-CSF and RANKL. Before the identification and purification of RANKL it was necessary to have RANKL presenting support cells in co-culture with myeloid cells (Hattersley and Chambers, 1989). In these cultures, addition of agents such as PGE<sub>2</sub>, dexamthasone, and vitamin D3 enhances osteoclast formation through indirect

actions on the supporting cells (Takahashi *et al.*, 1988). With the identification and purification of RANKL, osteoclasts can now be derived from non-adherent bone marrow cells of mice in the presence of M-CSF and RANKL (Yasuda *et al.*, 1998), with minimal contamination from stromal cells. A murine pre-osteoclast cell line, RAW 264.7 cells, is also used extensively as a source of osteoclasts. These cells are transformed murine macrophage precursors; they express RANK and have the potential to differentiate into bone-resorbing osteoclasts (Hsu *et al.*, 1999). They do not require additional M-CSF for differentiation as they express high levels of M-CSF protein and its receptor c-fms; considering the requirement for M-CSF observed in normal osteoclasts, interpretation of responses to treatments will always be clouded by this aberration (Collin-Osdoby *et al.*, 2003). If available, the use of 'normal' primary cells is always preferable to the use of transformed cells. These assays mimic events that occur *in vivo*, and allow the investigation of the mechanisms of osteoclast formation, and factors that can influence this process, in an easily observable manner. Together these assays allow an insight into how experimental factors can alter osteoclast formation and activity within genetically well-defined populations.

Hypoxia is known to stimulate the proliferation of haematopoietic cells (Bradley *et al.*, 1978; Ivanovic *et al.*, 2000). Hypoxia also causes acidosis due to increased anaerobic metabolism. Acidosis has been identified as the major stimulator of osteoclast activation (Arnett and Dempster, 1986). Stimulatory effects of hypoxia on osteoclast formation and activity were indicated by work performed previously in the lab. Incubation of murine calvariae in 2% O<sub>2</sub> led to 5-fold increases in calcium release, which was inhibited by the addition of indomethacin, a COX2 inhibitor. Furthermore, formation of osteoclasts from murine bone marrow cells was enhanced 3-fold at 2% O<sub>2</sub> compared to 20% O<sub>2</sub>. I furthered these studies by investigating the effects of oxygen tension on osteoclast formation across an oxygen range from atmospheric oxygen levels (20% O<sub>2</sub>) to severe hypoxia (0.2% O<sub>2</sub>). I then studied the activity of osteoclasts, as measured by resorption of dentine, across the same oxygen range.

## **Materials and methods**

### ***Reagents***

Culture medium and buffers were purchased from Gibco (Paisley, UK). Cylinders containing custom mixtures of O<sub>2</sub>, CO<sub>2</sub> and N<sub>2</sub> were purchased from BOC Gases (London, UK). M-CSF was a kind gift from the Genetics Institute (Cambridge, MA). All other reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

### ***Preparation of dentine discs***

Dentine slices were cut from untreated elephant ivory (a kind donation from HM Customs and Excise, Heathrow Airport, London, UK). 250µm thick transverse ivory (dentine) wafers were cut using a diamond saw (Isomet, Buehler Inc, Lake Bluff, IL), and soaked in deionised water before being cut into 5mm discs using a standard hole punch. Discs were then washed by sonication (10 x 5 minutes) in deionised water and stored dry. Discs were sterilised by brief immersion in ethanol, allowed to air dry and then washed in multiple changes of sterile phosphate buffered saline (PBS) before use in culture.

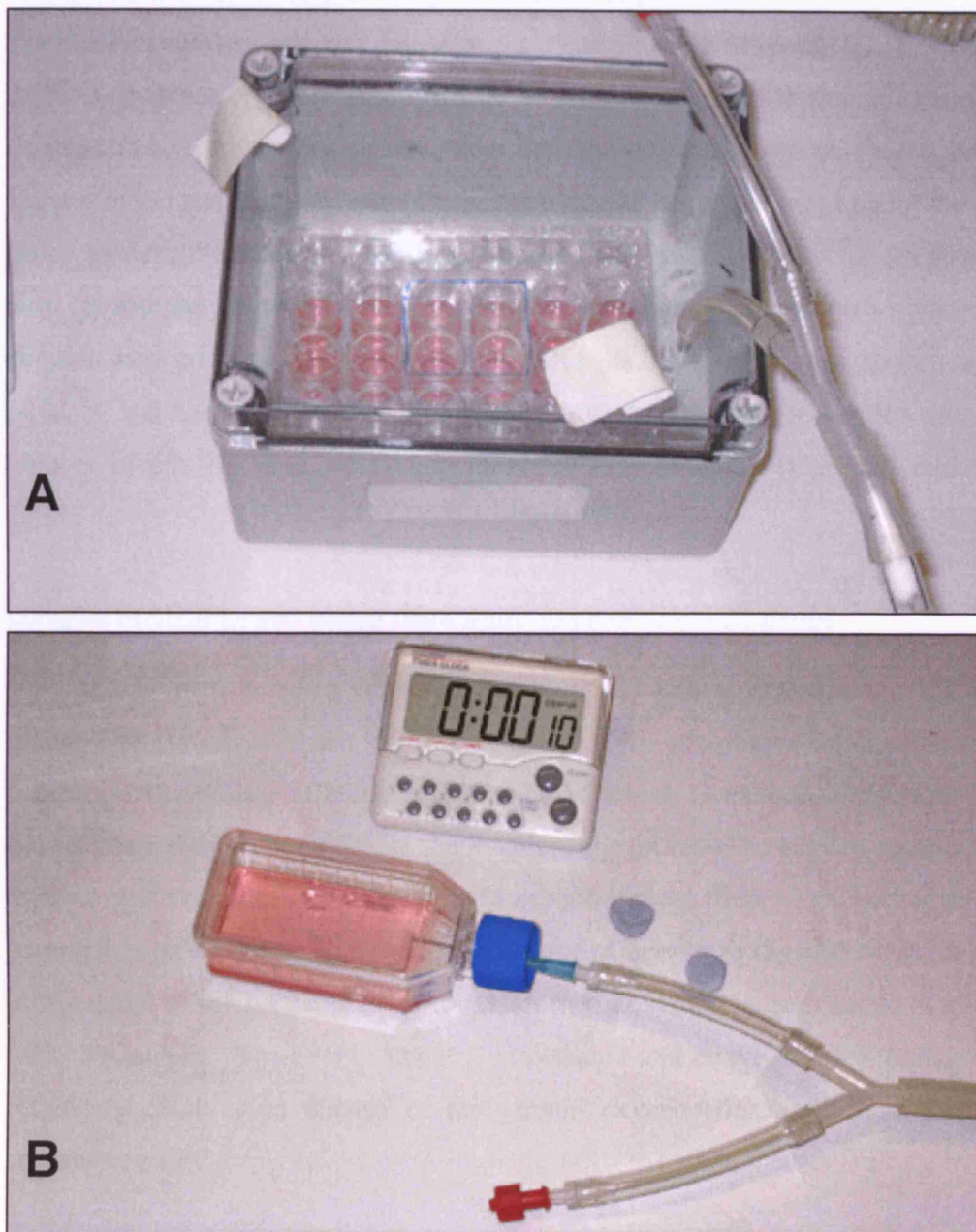
### ***Murine bone marrow osteoclast culture***

Long bones were isolated from one or two 6-8 week old MF1 mice killed by cervical dislocation. The epiphyses were removed and the marrow was flushed out with PBS using a 25-gauge needle. Marrow was collected and cells pelleted by centrifugation at 1000g, followed by a PBS wash, before being resuspended at  $5 \times 10^6$  cells / ml in minimal essential medium containing 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml amphotericin B (mixture abbreviated MEM) supplemented with  $10^{-8}$  M dexamethasone,  $10^{-7}$  M prostaglandin E<sub>2</sub> (PGE<sub>2</sub>),  $10^{-8}$  M 1,25-dihydroxyvitamin D<sub>3</sub>, (1,25(OH)<sub>2</sub>D<sub>3</sub>) and 20ng/ml M-CSF. Cells were placed into a 75 cm<sup>2</sup> tissue culture flask and incubated for 24 hours at 37°C / 5% CO<sub>2</sub> to allow attachment of stromal cells. Non-adherent cells were collected by centrifugation and resuspended at  $5 \times 10^6$  cells / ml in MEM containing  $10^{-8}$  M dexamethasone,  $10^{-7}$  M PGE<sub>2</sub>,  $10^{-8}$  M 1,25(OH)<sub>2</sub>D<sub>3</sub>, 20ng/ml M-CSF and 30 ng/ml RANKL. Dentine slices in 96 well trays were incubated with 200µl of cell

suspension ( $10^6$  cells) overnight at  $37^{\circ}\text{C}$  / 5%  $\text{CO}_2$  to allow attachment of osteoclast precursors to discs. Dentine discs were then transferred into  $25\text{ cm}^2$  tissue culture flasks with 'plug seal' caps (Falcon, Becton Dickinson, Oxford, UK) containing 8 ml of the same medium (8 discs per flask) and flasks purged with gas mixtures containing 20, 12, 5, 2, 1, or 0.2%  $\text{O}_2$  / 5%  $\text{CO}_2$  / balance  $\text{N}_2$  for 2 minutes through a 21 gauge needle inserted through the cap (**Figure 2.1**). Alternatively, discs were transferred into 24 well trays containing 2 ml of medium per well (2 discs per well) and placed into silicone vacuum grease-sealed, gas tight plastic fuse boxes, and gassed through holes drilled in the lid. Flasks and boxes were sealed and needles stopped with a Luer plug or 'Blutack', and incubated at  $37^{\circ}\text{C}$  / 5%  $\text{CO}_2$  in a humidified incubator. Medium was changed every 3-4 days and at day 12 was acidified to pH 7.0 by the direct addition of 12M HCl ( $82\mu\text{l}$  / 100ml). pH,  $\text{pCO}_2$ , and  $\text{pO}_2$  were monitored using a blood gas analyser (ABL 705, Radiometer, Copenhagen, Denmark), and  $\text{pO}_2$  levels below the measurement range of clinical blood gas analysers were measured using a fluorescence based fibre optic oxygen probe (FOXY, Ocean Optics, Duiven, Netherlands) as described below. Cultures were stopped 14 days post cell isolation. Ivory discs were removed from the flasks, PBS washed, then fixed in 2.5% glutaraldehyde for 3 minutes before staining for tartrate resistant acid phosphatase (TRAP) (Sigma Kit 387-A). The numbers of TRAP-positive multinucleate osteoclasts were assessed 'blind' using transmitted light microscopy. Discs were stripped of cells by sonication for 10 min in 0.2 M  $\text{NH}_4\text{OH}$  and then restained with toluidine blue to visualise resorption pits. The area resorbed per disc was quantified by dot-count morphometry under reflected light.

### ***Isolation of mature rat osteoclasts***

Mature osteoclasts were isolated as described previously (Hoebertz and Arnett, 2003). Briefly long bones were isolated from 2-day old Sprague-Dawley rat pups and placed into 5 ml MEM. Pooled long bones were rapidly minced and vortexed for 30 sec,  $100\mu\text{l}$  of the resulting cell suspension was placed onto pre-wetted dentine discs in a 96 well tray and allowed to sediment for 45 minutes. Discs were rinsed in PBS before being transferred into  $25\text{ cm}^2$  Falcon plug seal tissue culture flasks containing



**Figure 2.1: Apparatus for creation of tissue culture environments with controlled oxygen levels.**

**A:** Humidified, gas-tight plastic fuse boxes containing 24-well trays were purged daily for 2 minutes through holes drilled in the lid with gas mixtures containing 20, 12, 5, 2, 1, or 0.2% O<sub>2</sub> / 5% CO<sub>2</sub> / balance N<sub>2</sub> from custom formulated cylinders.

**B:** 25 cm<sup>2</sup> tissue culture flasks were gassed via a needle inserted through the cap. The polyethylene cap forms a gas tight seal against the needle, and the needle hub was closed with a Luer plug.

6ml of MEM acidified to pH 7.0 by the addition of concentrated HCl (6 discs / flask). The flasks were then gassed with mixtures containing 5% CO<sub>2</sub>, and 20, 12, 5, 2, 1, or 0.2% O<sub>2</sub> (balance N<sub>2</sub>), and incubated for 27 hours in a humidified atmosphere of 5% CO<sub>2</sub> / 95% air. At the end of the experiment the pH, pCO<sub>2</sub> and pO<sub>2</sub> were measured using a blood gas analyser, and pO<sub>2</sub> in the hypoxic range measured using the FOXY probe as described below. Total cell number was assessed by subsequent staining with 1% toluidine blue in 1% sodium borate for 2 minutes; all cells in 3 random fields per disc were counted. Discs were stripped of cells by sonication for 10 min in 0.2 M NH<sub>4</sub>OH and then restained with toluidine blue to visualise resorption pits. The number of pits per disc were counted under reflected light (Hoebertz and Arnett, 2003).

### ***Oxygen measurement using fibre optic oxygen (FOXY) probe***

Oxygen measurements in the hypoxic range were recorded using the FOXY probe. This functions in the following manner. The probe tip contains a ruthenium complex immobilised in a water resistant gel, which is excited by pulsed light at 475nm carried by an optical fibre the length of the probe. The excited complex emits light at ~600nm, which is detected by a second optical fibre. The excitation of the complex is quenched by non-radiative transfer of energy to O<sub>2</sub> molecules, at a level proportional to the partial pressure of O<sub>2</sub> in the gel, which is in dynamic equilibrium with the sample. The energy released is detected and converted to a digital signal, ultimately resulting in display of the sample oxygen concentration in the sensor software on a PC.

The FOXY probe and O<sub>2</sub> gas mixtures were calibrated by testing a gradient of oxygen tensions induced in medium lacking cells in sealed 25cm<sup>2</sup> flasks. Flasks were purged with gas mixtures for 90 seconds, followed by a further 90 second exposure 30 minutes later. Flasks were then incubated in a humidified incubator, supplied with 5% CO<sub>2</sub> and atmospheric air, at 37°C. 24 hours later oxygen tensions were measured in the flasks using the FOXY probe. As oxygen solubility is dependent on temperature the FOXY probe was calibrated at 37°C. **Table 2.1** shows the oxygen measurements taken over time for the relative gas mixtures.

Expected pO <sub>2</sub>	Time (minutes)					
	1	2	3	4	5	6
20%	24.6	22.6	22.0	20.9	<b>20.0</b>	20.0
12%	14.5	13.7	13.2	13.2	13.1	<b>13.0</b>
5%	7.4	6.8	6.6	<b>6.5</b>	6.6	6.9
2%	4.9	<b>3.9</b>	4.1	4.2	4.6	4.7
1%	4.8	4.0	<b>3.9</b>	4.1	4.1	4.2
0.2%	4.1	2.9	2.8	2.7	2.7	<b>2.6</b>

**Table 2.1: Recorded medium pO<sub>2</sub> 24 hours post-gassing; effect of time on measurement.**

Recorded pO<sub>2</sub>, as measured by FOXY probe, at 24 hours post gassing, in flasks containing MEM equilibrated to 20%, 12%, 5%, 2%, 1% or 0.2% O<sub>2</sub> / 5% CO<sub>2</sub> / balance N<sub>2</sub>. Readings were taken every minute following opening of flasks for insertion of probe, after 6 minutes readings remained unchanged or started to increase, suggesting equilibration with atmospheric O<sub>2</sub>; minimum values (indicated in **bold**) were obtained between 2-6 minutes.

Before testing of experimental medium samples the oxygen probe was calibrated to 37°C and 0% O<sub>2</sub> using a freshly prepared 7.5% w/v solution of the strong reducing agent sodium hydrosulphite (Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>) in PBS immersed in a 37°C water bath. The probe was left to equilibrate for several minutes until a stable reading was attained and then washed briefly before measuring O<sub>2</sub> in experimental samples. Flasks were taken from the incubator and a medium sample removed for pH testing by blood gas analyser. The lower half of each flask was immersed in a 37°C water bath and the probe inserted through the open top until submerged in medium, the reading was taken once the display had stabilised. Measurements were made with discs *in situ*, following oxygen measurement discs were removed from the flasks and processed as described above. Due to the smaller volume of medium in 24 well trays, oxygen measurements in silicone vacuum grease-sealed fuse boxes were performed using a bijou containing 2ml of medium as a surrogate sample. Bijous were placed in the boxes and the lid loosened to allow for gas exchange. Following opening of the fuse box the oxygen concentration in the bijou was measured using the FOXY probe as described for the 25cm<sup>2</sup> flasks. The FOXY probe was only utilised at the end point of experiments due to the difficulty of sterilising the oxygen probe.

### ***Statistical Analysis***

Statistical comparisons were made by one-way analysis of variance (ANOVA) using “Instat” software (version 1.3, Graph Pad, Inc, San Diego, CA); representative data are presented as means ± standard error of the mean (SEM). Significance was assumed at  $P < 0.05$ . Bonferroni adjustment was made for repeated comparisons. Results are presented for representative experiments that were repeated at least 3 times.



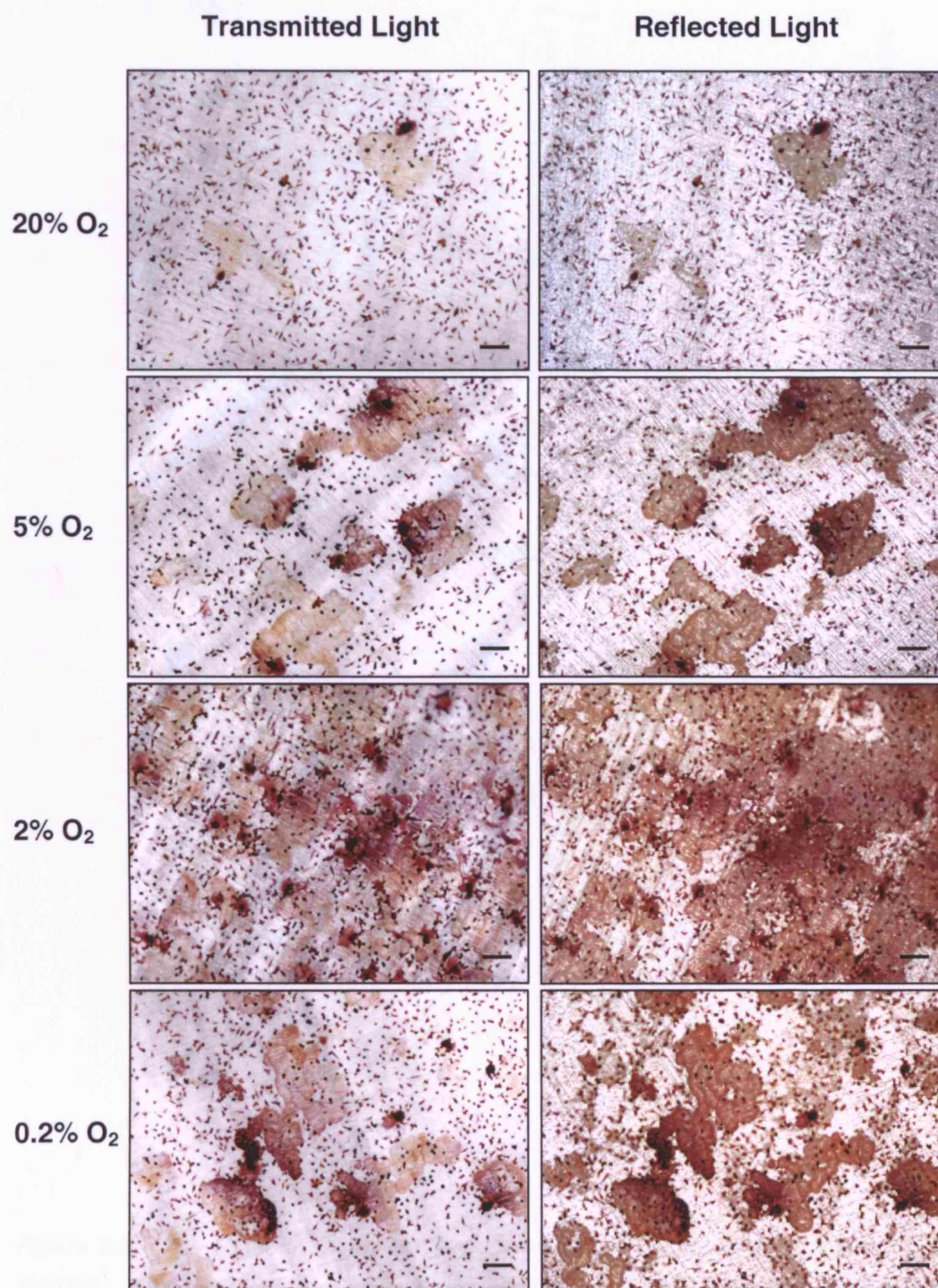
## Results

### *Experimental hypoxia in culture conditions*

It was of interest to know how quickly the oxygen tension would change in the experimental samples during measurement, as the probe could take up to 5 minutes to reach a steady value. The measurements indicate that the probe requires 2 - 6 minutes to attain its lowest value, with stable readings after approximately 6 minutes. There was a very slow change in medium pO<sub>2</sub> during the measurement period. Medium pO<sub>2</sub> was assumed to correspond to the lowest recorded values, which were as follows (expected value in brackets); 20% (20%), 13.1% (12%), 6.5% (5%), 3.8% (2%), 3.8% (1%) and 2.6% (0.2%). There was some leakage of atmospheric oxygen into the flasks and fuse boxes; this effect was greatest when pO<sub>2</sub> was very low, as would be expected over larger pO<sub>2</sub> gradients.

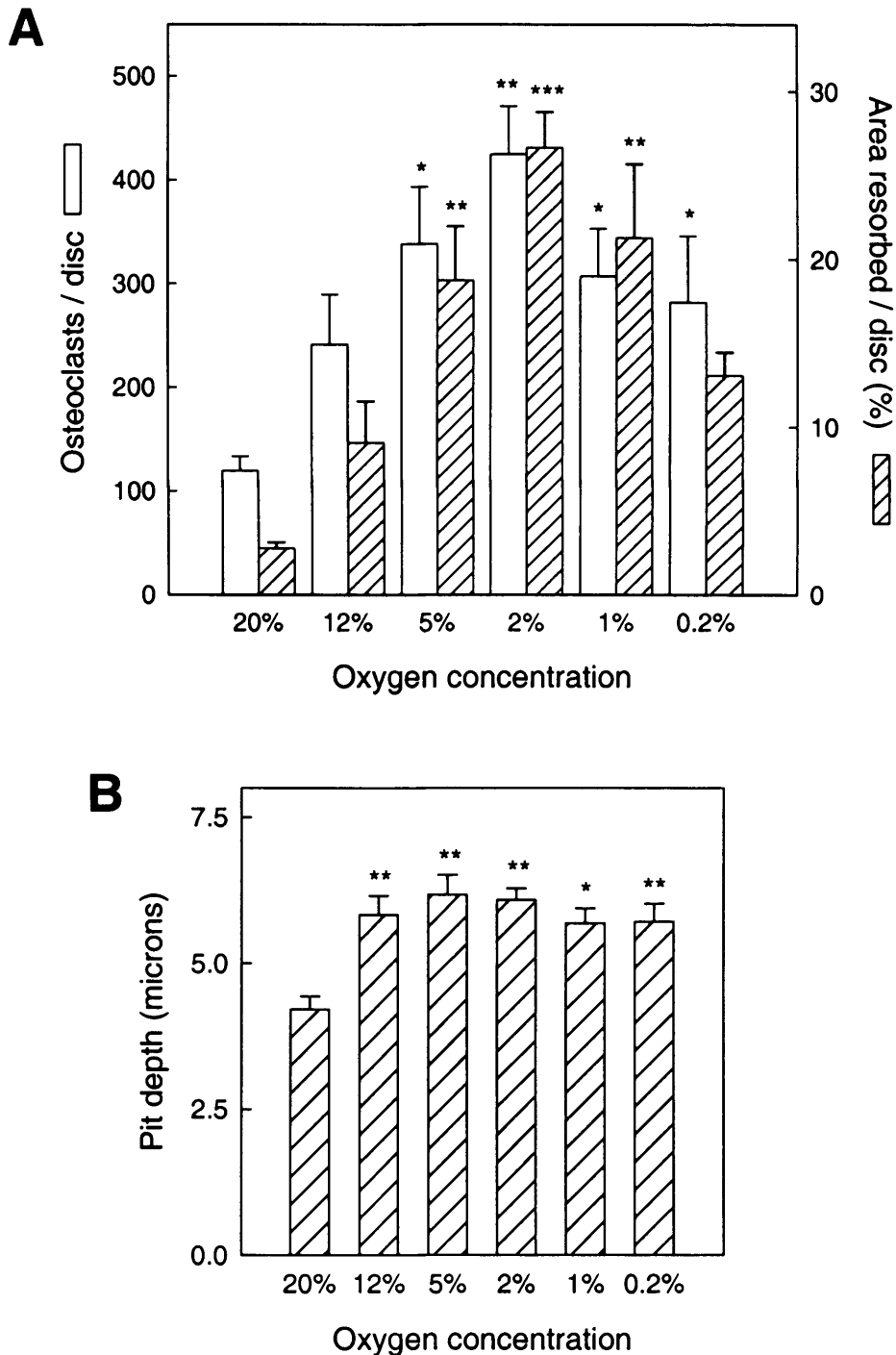
### *Hypoxia increases osteoclast formation from murine bone marrow*

Osteoclasts formed efficiently on dentine discs cultured in 20% O<sub>2</sub> over 12 days in the presence of permissive concentrations of RANKL & MCSF; however these cells were generally small, usually with 1-3 nuclei, and were identified as osteoclasts through the expression of TRAP and excavation of resorption pits (**Figure 2.2**). In comparison, cells cultured in 2% O<sub>2</sub> were more numerous and larger in appearance. When osteoclast numbers were quantified it was evident that peak osteoclast formation occurred at the 2% O<sub>2</sub> level (**Figure 2.3a**). At this oxygen tension osteoclast number increased approximately 4-fold; however even severe hypoxia (0.2% O<sub>2</sub>) caused a significant, 2-fold increase in osteoclast number. Even a slight decrease in oxygen tension to 12% was sufficient to increase the osteoclast numbers markedly. The amount of resorption occurring in lower oxygen tensions was increased when expressed as a percentage of the entire disc resorbed, and when calculated as the area resorbed per osteoclast (**Figure 2.3a**). This was consistent with the observed size increase in osteoclasts formed in low oxygen cultures. Additionally, the pits excavated by osteoclasts formed in 20% O<sub>2</sub> (hyperoxia) were



**Figure 2.2: Stimulation of osteoclast formation and resorption in murine bone marrow cultures by hypoxia.**

Osteoclast formation on dentine discs induced by RANKL and M-CSF was detected by TRAP staining. Osteoclast formation increased as oxygen tension decreased with corresponding increases in resorption. (scale bar = 100 $\mu$ m)



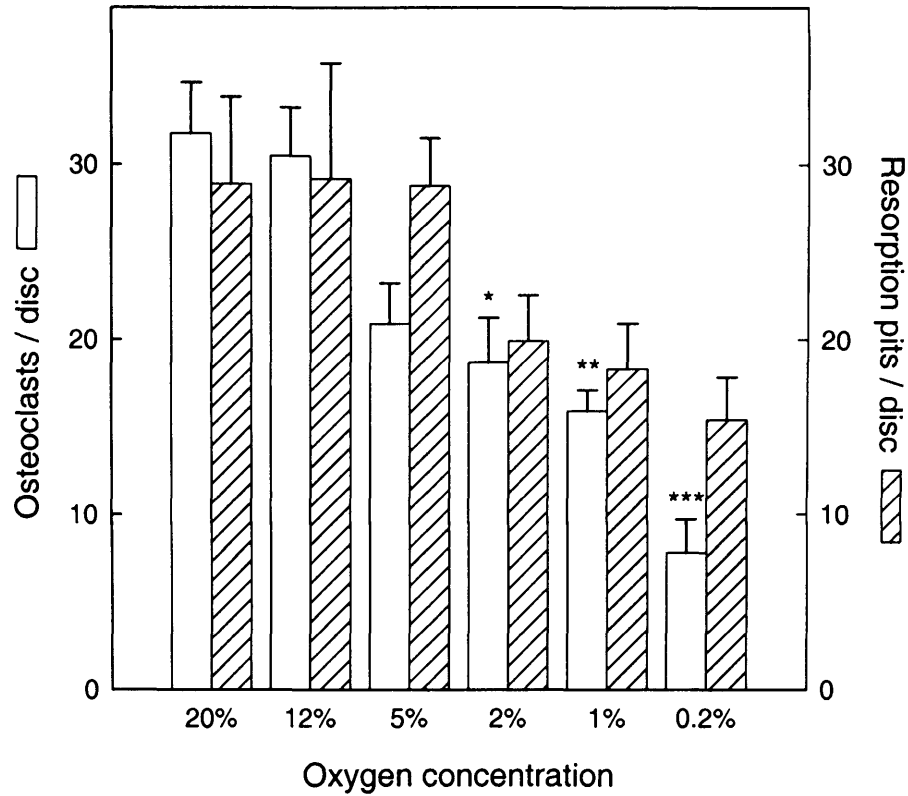
**Figure 2.3 A: Hypoxia increases the formation of osteoclasts from murine bone marrow.** Peak formation occurred in 2% O<sub>2</sub>, with significant stimulation even in severe hypoxia (0.2% O<sub>2</sub>). Total resorption of dentine increased in line with the increases in osteoclast formation. Values are mean  $\pm$  standard error; n = 6; \* p<0.05, \*\* p<0.01, \*\*\* p<0.001 vs. 20% O<sub>2</sub>.

**B: Resorption pits excavated by osteoclasts formed in hyperoxia (20% O<sub>2</sub>) are shallower than those formed in lower oxygen tensions.** The average depth of resorption pits was significantly deeper when osteoclasts were formed in oxygen concentrations below 20% O<sub>2</sub>. Values are mean  $\pm$  standard error, n = 10; \* p<0.05, \*\* p<0.01 vs. 20% O<sub>2</sub>.

shallower than those formed in lower oxygen tensions, which leads to an approximate 3-fold decrease in volume resorbed per pit (**Figure 2.3b**).

***Hypoxia does not activate resorption in mature rat osteoclast cultures***

To investigate whether hypoxia is capable of directly activating mature osteoclasts, a rat osteoclast assay was employed. As the oxygen tension decreased, the osteoclast number fell; indicating that the survival or attachment to dentine of mature rat osteoclasts was decreased following incubation in low oxygen conditions for 27 hours (**Figure 2.4**). The decrease in osteoclast number was accompanied by a decrease in resorption. The number of pits formed per osteoclast was increased 2-fold in 0.2% O<sub>2</sub>, but was unchanged at higher oxygen tensions relative to 20% O<sub>2</sub>.



**Figure 2.4: Hypoxia decreases survival or attachment of mature rat osteoclasts.** Mature rat osteoclasts exposed to levels of  $O_2$  below 5% exhibited a decrease in their survival or attachment to dentine indicated by a significant drop in osteoclast number per disc after 27 hours of culture. The number of resorption pits per disc decreased as osteoclast number decreased, with the number of pits per osteoclast relatively unchanged, however at 0.2%  $O_2$  the number of resorption pits was approximately 2-fold increased relative to osteoclast number compared to 20%  $O_2$ . \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. 20%  $O_2$ . Values are mean  $\pm$  standard error;  $n = 10$ .

## Discussion

My work has confirmed initial observations that hypoxia stimulates the formation of osteoclasts from murine bone marrow cells cultured in the presence of RANKL and M-CSF. It also revealed that hypoxia has detrimental effects on mature osteoclast survival or attachment to dentine.

The method used to create a hypoxic environment in these experiments was chosen because it allowed a large range of oxygen tensions to be investigated, whilst keeping other parameters such as temperature as consistent as possible between treatment groups (i.e., cultures at different oxygen tensions are maintained in a single incubator). The FOXY probe is an accepted method of oxygen measurement in tissue culture samples because it avoids the interference caused by pH and protein levels, and does not consume oxygen, as is the case for other sensor types (for example, Clark electrodes) (Allen *et al.*, 2001). The oxygen tension in the gas phase of culture vessels reaches equilibration with the input gas level following several minutes of purging; however, limitations to oxygen diffusion in medium result in a 2-3 hour delay in equilibration of the medium with the gas phase (Allen *et al.*, 2001). Although some leakage of atmospheric oxygen back into flasks and boxes occurred, the measured pO<sub>2</sub> after 24 hours indicated that the oxygen levels obtained in the hypoxic cultures were approaching the desired levels.

I have shown that osteoclast numbers increase when marrow precursor cells are incubated in hypoxic environments. This increased formation could be due to a number of factors. Hypoxia has been shown to promote the proliferation and maintenance of pluripotency of haematopoietic stem cells, and to increase the proliferation of committed bone marrow-derived haematopoietic cells (Bradley *et al.*, 1978; Ivanovic *et al.*, 2000; Ivanovic *et al.*, 2004). It is possible that proliferation of monocytic precursors increased in hypoxic cultures, leading to increased osteoclastogenesis. However, since average marrow pO<sub>2</sub> is thought to be about 6.5%, at least in normal humans (Harrison *et al.*, 2002; Ishikawa and Ito, 1988), it must be borne in mind that oxygen concentrations of 20% (i.e., atmospheric) and 12% in fact constitute *hyperoxia*. Considered from this perspective, the inhibitory action

of hyperoxia is at least as great as the stimulatory action of hypoxia. Increased production of reactive oxygen species may be limiting the osteoclastic potential or survival of cells in atmospheric oxygen. I attempted to address this question by adding the antioxidant, ascorbic acid, to cultures (at 50 µg/ml, the concentration used to promote collagen production by osteoblasts); surprisingly, however this resulted in extensive cell death at all oxygen tensions (data not shown).

Another possibility is that more myeloid lineage precursors were induced to differentiate into osteoclasts in hypoxia. This could be occurring through direct effects of hypoxia on preosteoclasts, possibly mediated through HIF stabilisation (please see Chapter 3). Although stromal cell 'contamination' in the murine bone marrow cell culture system used here is low (Yasuda *et al.*, 1998), it is possible that any such cells present could respond to hypoxia by increasing their production of the osteoclastogenic cytokines RANKL and M-CSF. It is also possible that increases in expression of receptors for these factors on osteoclast precursors in hypoxia could be involved in mediating osteoclastogenesis. There are other osteoclastogenic factors produced by both stromal cells and monocytic cells that are induced by hypoxia in other cell types. These include the inflammatory mediators PGE<sub>2</sub> (Cernanec *et al.*, 2002; Saed *et al.*, 2005), TNFα (Lewis *et al.*, 1999), IL-1 and IL-6 (Ala *et al.*, 1992), and the angiogenic cytokine vascular endothelial growth factor (VEGF) (Akeno *et al.*, 2001; Steinbrech *et al.*, 1999; Steinbrech *et al.*, 2000). Recent studies in our laboratory have shown that hypoxia stimulates resorption in cultured calvarial bones up to 5-fold; this effect was prevented by the cyclooxygenase inhibitor, indomethacin, suggesting that the response was mediated via prostaglandin production (Arnett *et al.*, 2003). The effect of hypoxia on TNFα and IL-6 protein expression in osteoclast-forming human peripheral blood mononuclear cell cultures is presented in Chapter 3.

To date the only other published work on the action of hypoxia on osteoclastogenesis is the recent study of Fukuoka and colleagues (2005). This group investigated the effects of hypoxia on gene expression in murine marrow cultures using microarray technology. They found that hypoxia increased the expression of insulin-like growth factor 2 (IGF2) in stromal cells present in murine bone marrow cultures, but not in osteoclasts. Addition of exogenous IGF2 in normoxia stimulated

osteoclastogenesis to levels similar to those observed in hypoxic cultures, leading to the conclusion that osteoclast formation in hypoxia could be mediated indirectly via stromal cell production of IGF2. However, Treins and colleagues (2005) recently showed that exogenous IGF1 acts to stabilise HIF1 $\alpha$  in normoxia, at least in retinal epithelial cells and human embryonic kidney cells. It is therefore potentially possible that IGF2 might elicit a similar stabilisation of HIF1 $\alpha$  in murine osteoclast cultures, resulting in increased osteoclastogenesis. Microarray or other mRNA analysis would not detect this possibility, as HIF1 $\alpha$  message is constitutively expressed. Protein levels of HIF $\alpha$  subunits are regulated post-transcriptionally; I am currently investigating the expression and hypoxic stabilisation of HIF1 $\alpha$  and HIF2 $\alpha$  proteins in osteoclasts exposed to hypoxia. It is also possible that HIF signalling could induce the production of IGF by stromal cells, since IGF is a target gene for HIF in osteoblasts (Steinbrech *et al.*, 2000).

My results indicate that the survival or attachment of mature cells in short-term cultures was compromised when oxygen concentration was below 5%. From these results, it is not possible to ascertain whether the decreased number of mature osteoclasts observed after 27 hours exposure to hypoxia was due to increased osteoclast apoptosis or to decreased attachment to the substrate. However, the data indicate that osteoclasts were able to survive/adhere for long enough in severe hypoxia (0.2% O<sub>2</sub>) to produce resorption pits. These results suggest that the increased osteoclastogenesis (and thus resorption) observed in long-term hypoxic marrow cultures could occur against a background of reduced survival/adhesion of mature osteoclasts – in other words, a ‘live fast, die young’ effect. A simple way to investigate these differing outcomes is to look for apoptotic osteoclasts after 4-5 hours in culture using TdT-mediated dUTP-dioxigenin nick end labelling (TUNEL); by this stage apoptosis induced by hypoxia should be easily detectable.

A known consequence of hypoxia is acidosis (please see Chapter 1), a potent stimulator of osteoclast activity (Arnett and Dempster 1986; Arnett and Spowage, 1996). Care was taken whilst performing my investigations that medium pH was not affected by reduced oxygen tension. In the earlier experiments examining the effects of hypoxia on calvaria (Arnett *et al.*, 2003), we found that hypoxia caused slight



acidification of the culture medium and this may have been accompanied by localised acidosis within the bone environment. This could help to account for the observed increase in calcium release in calvarial organ cultures exposed to hypoxia. Acid induced  $\text{Ca}^{2+}$  release in organ culture is also blocked by indomethacin (Goldhaber and Rabadjija, 1987; Meghji *et al.*, 2001). *In vivo* hypoxia causes acidosis due to increased anaerobic metabolism and decreased perfusion. Therefore, although hypoxia did not induce osteoclast activation directly, it would activate resorption indirectly due to the acidosis accompanying it *in vivo*.

It has been shown that disuse induces osteocyte hypoxia and HIF stabilisation (Gross *et al.*, 2001). Several studies have examined the effects of decreased blood supply to rodent bones by unloading the hind limbs; this assay is also used to study the effects of disuse on bone loss. Colleran and colleagues (2000) demonstrated that hind-limb unloading led to alterations in bone perfusion, with reduced blood flow to the femur and tibia. This was correlated with reduced trabecular and cortical bone mass in these bones. In contrast, the mandible and clavicle received increased blood flow, and this correlated with increased bone mass. In the study by Grano *et al.* (2002) hind-limb unloading led to thinning of the trabeculae in unloaded bones, and increased osteoclast formation in *ex vivo* cultures from unloaded bones. This suggests that the effects of hypoxia may be cell-autonomous, as *ex vivo* osteoclast cultures were exposed to 20%  $\text{O}_2$  in these studies. Although no measurements were made of blood  $\text{pO}_2$  or pH, these results give an indication of the potential effects of altering the blood supply to the bone, and are broadly consistent with my observations on the effects of hypoxia on osteoclast activity.

Having established that the effects observed in preliminary investigations are consistent, and quantified the extent of the response in marrow derived, and mature osteoclast cultures in rodent models, I then investigated the effects of hypoxia on human cells derived from peripheral blood mononuclear cells.

# CHAPTER 3

## *Hypoxia stimulates the formation of osteoclasts from human peripheral blood*

### **Introduction**

Although rodent models provide an insight into the processes occurring during osteoclast formation, it is important to be able to investigate and verify effects in human systems. Prior to the identification of RANKL, the isolation of human osteoclasts was difficult. Mature osteoclasts could be isolated from giant cell tumours of bone, also termed osteoclastomas, and utilised in studies of osteoclast activation (Chambers *et al.*, 1985). Experience in our own laboratory has shown that these cells display abnormally high resorptive activity, perhaps reflecting their tumour origins. The use of murine bone marrow led to attempts to use human bone marrow as a source of osteoclast precursors (Flanagan *et al.*, 1992; MacDonald *et al.*, 1987); however, human marrow is difficult (and painful) to extract. The discovery of RANKL, and subsequent generation of recombinant forms, allowed the generation of purer populations of human osteoclasts from easily accessible human peripheral blood mononuclear cells (hPBMNC) (Matsuzaki *et al.*, 1998).

Monocytic osteoclast precursors are isolated from blood by simple density gradient centrifugation. Further purification of monocytes using CD14 positive cell sorting has been used to ensure freedom from contaminating lymphocytes (Massey and Flanagan, 1999). An advantage of this assay system over marrow cultures is the comparative purity of the population relative to stromal cells. A recent but unconfirmed report indicated that there may be mesenchymal precursors circulating

in the peripheral blood, however their numbers are very low compared to the overwhelming numbers of monocytic cells (Eghbali-Fatourehchi *et al.*, 2005).

The hPBMNC culture system also offers the opportunity to study activation of mature osteoclasts due to the ability to control osteoclast activation using medium pH. Studies in our laboratory have shown that maintenance of medium pH at 7.45 is sufficient to prevent human osteoclast activation (Brandao-Burch and Arnett, 2004). By maintaining pH at this level until day 12 of culture it is possible to generate mature, non-resorbing osteoclasts. Subsequent lowering of the pH switches resorption on and the effect of treatments on osteoclast activity can then be measured.

My work on rodent osteoclasts (Chapter 2; Arnett *et al.*, 2003) provided clear evidence that oxygen tension can regulate osteoclast formation, but the mechanisms by which this occurs were not clear. The aim of the work presented in this chapter was to determine if the osteoclastogenic response to hypoxia is conserved in human osteoclasts, and if so, to investigate the kinetics and mediators of this response.

## **Materials and methods**

### ***Reagents***

Dentine discs were prepared as described in Chapter 2. RANKL was purchased from R&D Systems, M-CSF was a gift from the Genetics Institute (Cambridge, MA, USA). Anti HIF-1 $\alpha$  antibodies were purchased from BD Transduction labs (Oxford, UK), and Santa Cruz Biotechnology (Santa Cruz, CA, USA) and rabbit anti-HIF2 $\alpha$  (PM9) was a kind gift from Professor Patrick Maxwell (Imperial College London). FITC-conjugated and HRP-conjugated secondary antibodies were purchased from DAKO (Ely, UK). Unless otherwise stated all tissue culture medium and supplements were purchased from Gibco (Paisley, UK), and chemicals were purchased from Sigma (Poole, UK). Gas mixtures were purchased from BOC gases (London, UK).

### ***Human peripheral blood mononuclear cell cultures***

Blood was taken from healthy volunteers, and peripheral blood mononuclear cells (PBMNC) were isolated by density gradient separation, essentially according to the method described by Massey and Flanagan (1999). PBMNC were washed once in PBS and then sedimented onto dentine discs in 96 well trays (Iwaki, Chiba, Japan) in MEM / 15% heat inactivated FCS / 100 U/ml penicillin / 100  $\mu$ g/ml streptomycin / 2mM L-glutamine (mixture abbreviated to 'MEM'). After 4 hours, discs were washed vigorously in PBS before being placed into 96 well trays in MEM containing 5 – 10 ng/ml M-CSF. After 4 days incubation, discs were transferred to 25 cm<sup>2</sup> flasks or 24 well trays containing MEM with the pH adjusted to 7.45 by the addition of 7.5 meq NaOH, and further supplemented with 5-10 ng/ml M-CSF and 1 ng/ml RANKL. Required oxygen tensions were obtained by purging flasks and 24 well trays in humidified, gas tight fuse boxes with gas mixtures containing 20 – 0.2% O<sub>2</sub>, 5% CO<sub>2</sub>, balance N<sub>2</sub> as described in Chapter 2. Half of the medium was replaced every 3-4 days and medium was acidified to pH 7.0 by the addition of 10 meq HCl 48 hours prior to termination of the culture. Cells were fixed in 2.5% glutaraldehyde and stained for tartrate resistant acid phosphatase (TRAP) using the leukocyte acid

phosphatase kit (387-A, Sigma, Poole, UK) according to the manufacturer's instructions.

### ***Enzyme linked immunosorbent assay (ELISA)***

Medium was harvested from human osteoclast cultures grown on dentine discs and analysed for the presence of IL-6 and TNF $\alpha$ . Sandwich ELISA sets were purchased from Immunotools (Friesoythe, Germany) and were performed according to the manufacturers instructions. Briefly Nunc Maxisorp 96 well trays (VWR, Lutterworth, UK) were coated overnight with coating antibody. Wells were blocked with PBS/5% milk and then diluted culture medium samples added. The biotinylated secondary antibody was added and incubated with OPD substrate (Sigma, Poole, UK), upon colour development the reaction was stopped with 1M H<sub>2</sub>SO<sub>4</sub> and absorbance read at 405nm; cell debris was excluded by reading absorbance at 490nm.

### ***Reverse transcriptase polymerase chain reaction (RT-PCR)***

RNA for RT-PCR was isolated from osteoclasts grown on dentine discs in gassed 25cm<sup>2</sup> tissue culture flasks. Discs were washed briefly in PBS, and 1ml of TRIzol reagent (Invitrogen, Paisley, UK) was added to each flask and processed according to the manufacturer's instructions. The RNA pellet was dissolved in 50  $\mu$ l of DEPC treated water; 4  $\mu$ l of total RNA was utilized for cDNA production using the ImPromII Reverse Transcription System (Promega, Southampton, UK), according to the manufacturer's instructions. cDNA levels were normalized to beta-actin expression and used in polymerase chain reactions using primer pairs listed in **Table 3.1**.

### ***Western blotting***

Crude nuclear extracts were obtained from human osteoclast cultures grown on plastic in 6 well trays, following 6 or 24-hour exposure to 0.2, 2 or 20% oxygen in gas-tight boxes. Briefly, cells were washed twice in ice-cold PBS and then 300 $\mu$ l of Buffer A (10mM HEPES (pH 7.9) / 10mM KCl / 0.1mM EDTA / 1mM DTT /

Gene	Forward	Reverse
Beta actin	CAG AGC AAG AGA GGC ATC	GAG GTA GTC AGT CAG GTCC
M-CSF	GAT CCA GTG TGC TAC CTT	GTA CAG GCA GTT GCA ATC
c-fms	GAT GAG TTC CTC TTC ACA C	CTC ATG ATC TTC AGC TCG
RANK	GGC ACT GGA TCA ATG AGG	GCC TGT CCA TGT ATT CAT
TNF $\alpha$	CAG GCA GTC AGA TCA TCT TC	CTT GGA CTG GTA GGA GAC G
IL1 $\beta$	GTG TCT GAA GCA GCT ATG	GAG GTG GAG AGC TTT CAG
VEGF	GAG ACC CTG GTG GAC ATC T	CAC CGC CTT GGC TTG TCA C
HIF1 $\alpha$	CAG AAA TGG CCT TGT GAA	CAG GCT GTG TCG ACT GAG
HIF2 $\alpha$	GAT AGC AAG ACC TTC CTG	CAA AGA TGC TGT TCA TGG

**Table 3.1: Table of primer pairs used for human osteoclast cDNA amplification.**

0.5mM PMSF / 0.4% Igepal / 1µg/ml protease inhibitor cocktail) was added to each well and incubated at room temperature for 10 minutes. Cells were scraped off plastic and transferred into a 1.5 ml Eppendorf tube prechilled to 4°C. Lysate was centrifuged at 15,000g for 3 minutes to pellet nuclei and the supernatant was discarded. Nuclei were lysed by resuspension in 100µl of Buffer B (20mM HEPES (pH 7.9) / 0.4M NaCl / 1mM EDTA / 10% glycerol / 1mM DTT / 0.5mM PMSF / 1µg/ml protease inhibitor cocktail) followed by vigorous shaking for 2 hours at 4°C. Lysates were clarified by centrifugation at 4°C for 5 minutes at 15,000g. Nuclear protein extract was stored at -80°C. Proteins were separated by SDS-PAGE on an 8% resolving gel. Separated proteins were transferred onto a PVDF membrane (Amersham) using a Biorad MiniProtein II wet blotting apparatus at 150V for 1 hour, according to the manufacturer's instructions. Membranes were blocked by incubation for 1 hour in 5% non-fat milk / PBS, with shaking. Primary antibody (mouse anti-HIF1α, BD Transduction Labs) was diluted 1:250 in 2% non-fat milk / PBS and incubated overnight with membrane, at room temperature with shaking. The membrane was washed briefly in dH<sub>2</sub>O followed by three 15 minute washes in 0.05% Tween-20 / PBS. Secondary antibody (HRP-conjugated Rabbit anti-mouse immunoglobulins, Dako) was diluted 1:2000 in 1% non-fat milk / PBS and added to the membrane for 1 hour at room temperature. The membrane was washed and then incubated with ECL reagent (1.25mM luminol, 0.2mM coumaric acid, 0.1M Tris pH 8.5, 0.032% H<sub>2</sub>O<sub>2</sub>); signal was detected by exposure to Hyperfilm (Amersham). Even loading of protein was determined by Ponceau staining (0.5% w/v Ponceau, 1% v/v acetic acid, 200µM NaOH, 20% v/v acetonitrile) of the membrane.

### ***Immunofluorescence staining of human osteoclasts***

Osteoclasts grown on dentine discs were fixed in 3.7% paraformaldehyde in PBS for 8 minutes at room temperature. Cells were permeabilised by incubating in permeabilisation buffer (0.5% Triton X-100, 300mM sucrose, 50mM NaCl, 3mM MgCl<sub>2</sub>, 0.05% NaN<sub>3</sub>) for 5 minutes on ice. Non-specific staining was blocked by incubating in 3% BSA / PBS for 30 minutes at 37°C. Discs were then immersed in primary antibody diluted in 3% BSA / PBS (BD mouse anti- HIF1α – 1/1000; PM9 rabbit anti-HIF 2α - 1/1000) for 1 hour at room temperature. Discs were washed 3

times in PBS for 5 minutes before immersing in FITC conjugated secondary antibody diluted in 3% BSA / PBS (Dako, 1/30 – 1/40) for 45 minutes in the dark. Cells were stained for 30 minutes with rhodamine phalloidin to visualise F-actin rings.

### ***Statistical Analysis***

Statistical comparisons were made by one-way analysis of variance (ANOVA) using “Instat” software (version 1.3, Graph Pad, Inc, San Diego, CA); representative data are presented as means  $\pm$  standard error of the mean (SEM). Significance was assumed at  $P < 0.05$ . Bonferroni adjustment was made for repeated comparisons. Results are presented for representative experiments that were repeated at least 3 times.



## Results

### *Hypoxia stimulates the formation of human osteoclasts*

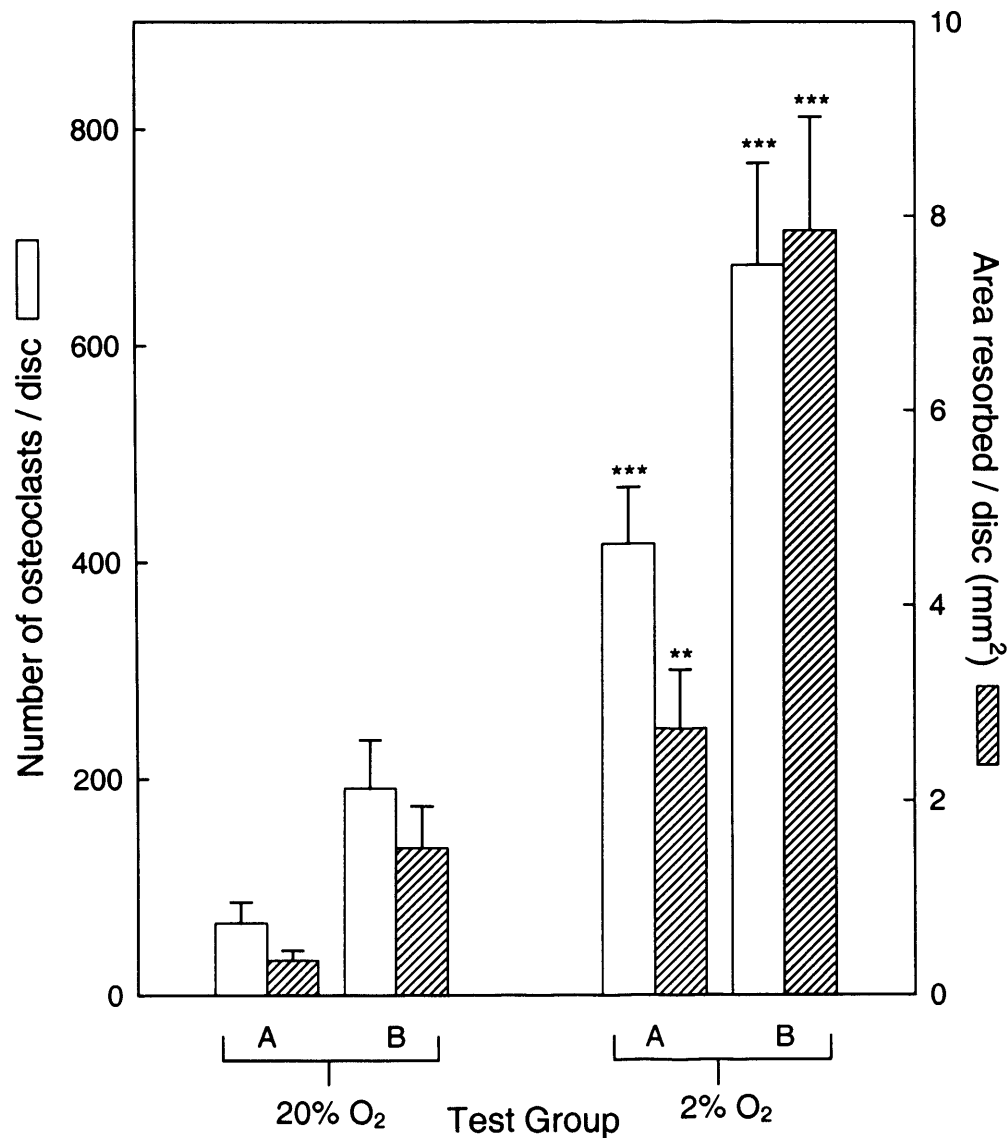
Basal osteoclast formation from human peripheral blood varied between donors, although a consistent 2 to 6 fold increase in osteoclast formation was observed when cells were exposed to 2% O<sub>2</sub> (**Figure 3.1**). Resorption was also increased by osteoclasts formed in 2% O<sub>2</sub>; however these cells were bigger. Osteoclasts formed in 2% O<sub>2</sub> averaged 3.3 nuclei per osteoclast whereas those formed in 20% O<sub>2</sub> averaged only 1.8 nuclei per osteoclast; thus, many of the functional osteoclasts in these cultures were mononuclear (**Figure 3.2**). Osteoclast formation peaked in 1 – 2% O<sub>2</sub>; however, even severe hypoxia (0.2% O<sub>2</sub>) significantly stimulated osteoclast formation (**Figure 3.3**). Osteoclast numbers were unaffected by 3 days of hypoxia (2% O<sub>2</sub>) following 14 days in 20% O<sub>2</sub>, and resorption was not activated by hypoxia in the absence of an acid stimulus (**Figure 3.4**). All cells in human PBMNC cultures stained TRAP positive; there was no evidence of fibroblastic colony formation regardless of pO<sub>2</sub> or length of time in culture.

### *Hypoxia increases the rate of osteoclast formation in long-term cultures*

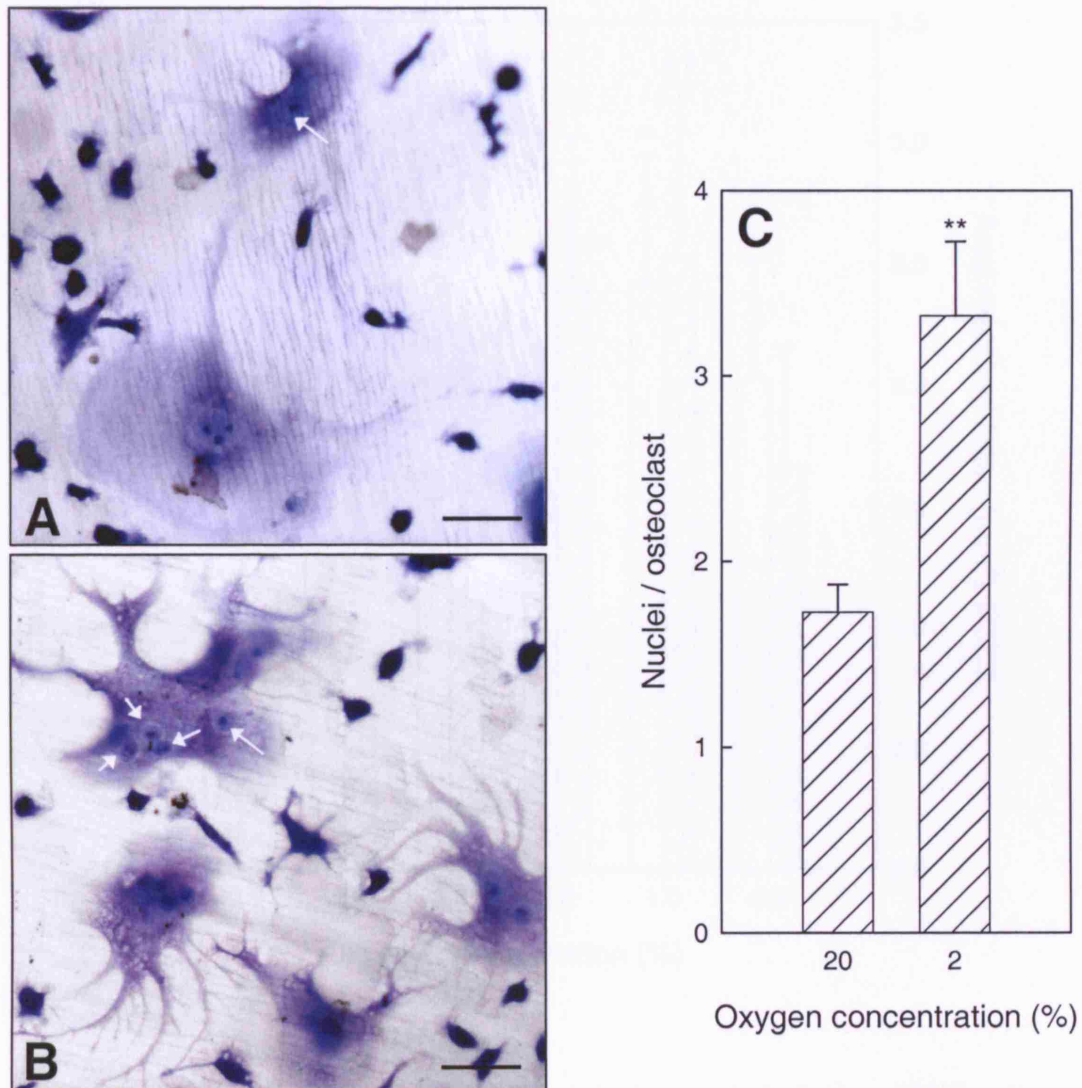
Extended osteoclast cultures (28 days) indicated that hypoxia increases the rate at which osteoclasts formed. Osteoclasts were not observed prior to day 7 in either 20% or 2% O<sub>2</sub> cultures (**Figure 3.5**). Hypoxia exerted a significant trophic effect on osteoclast numbers in long-term cultures. Osteoclast number continued to rise throughout the 28 day culture period, resulting in 4.5-fold increases in osteoclast number at day 28 of culture in 2% O<sub>2</sub> compared to 20% O<sub>2</sub>.

### *The hypoxia effect is dependent upon permissive levels of M-CSF and RANKL*

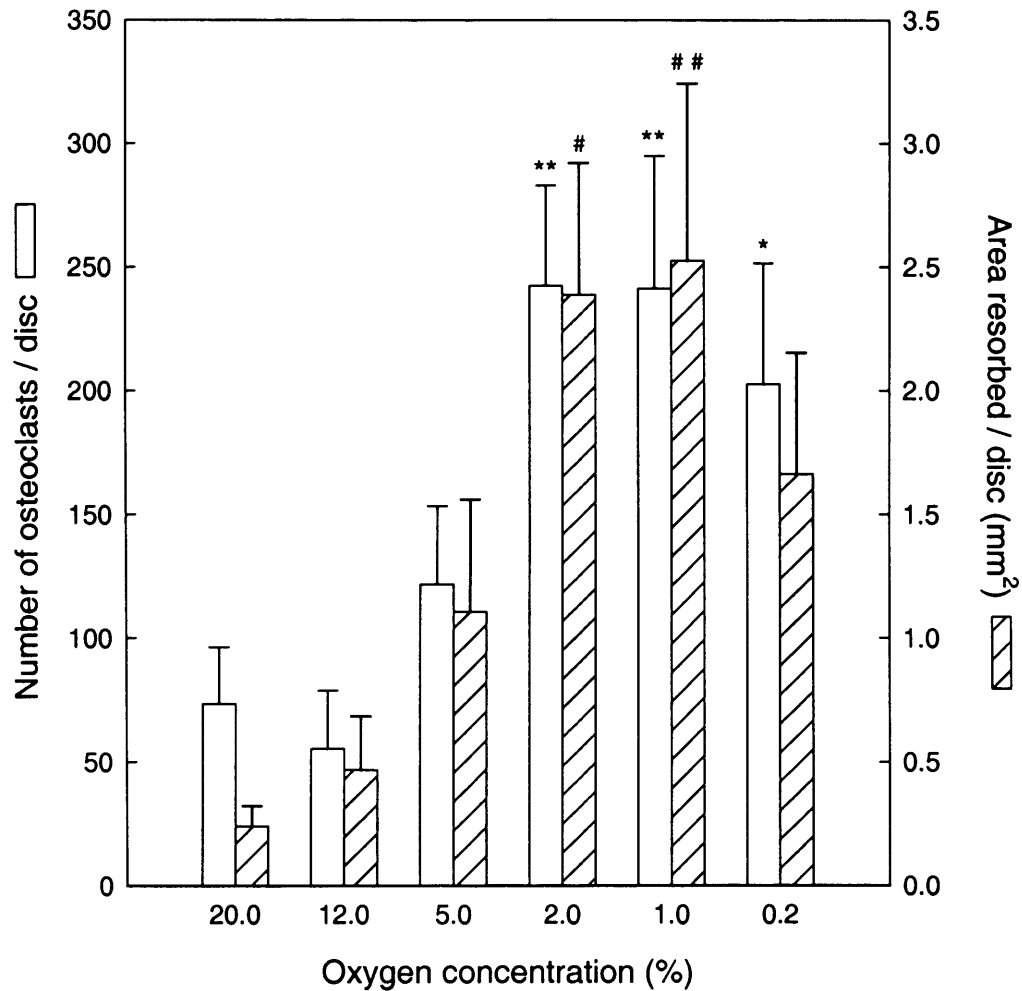
When PBMNC were cultured in MEM containing 1 ng/ml RANKL but lacking M-CSF no osteoclasts formed. This deficit could not be rectified by culturing cells in 2% O<sub>2</sub> (**Figure 3.6A**). When the amount of M-CSF was increased hypoxia was capable of stimulating osteoclast formation; osteoclasts formed in 2% O<sub>2</sub> were more



**Figure 3.1: Hypoxia stimulates human osteoclast formation from peripheral blood mononuclear cells.** The chart show typical responses of mononuclear cells isolated from 2 separate donors (A and B) and exposed to 20% or 2% O<sub>2</sub>. Basal osteoclast formation differed between donors; hypoxia stimulated osteoclast formation 6-fold and 3-fold, respectively in A and B. Osteoclastic resorption was also increased by culture in hypoxic conditions. \*\* p < 0.01, \*\*\* p < 0.001 vs. 20% O<sub>2</sub>. Values are means +/- SEM; n = 8.

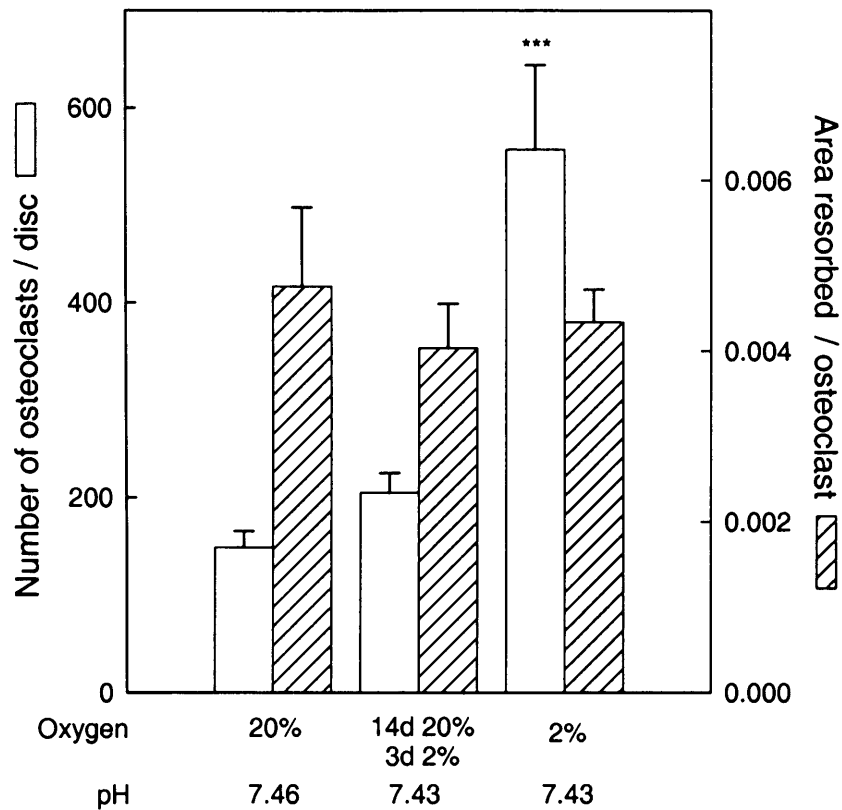


**Figure 3.2: Osteoclasts formed in 2% O<sub>2</sub> contain more nuclei than those formed in 20% O<sub>2</sub>.** **A:** Transmitted light image of toluidine blue-stained human osteoclasts formed in 20% O<sub>2</sub> (hyperoxia), showing the formation of small osteoclasts (1 - 2 nuclei); the white arrow indicates a mononuclear osteoclast. **B:** In contrast, hypoxia induced the formation of larger osteoclasts (3 - 4 nuclei). (White arrows indicate nuclei; scale bar = 50µm) **C:** Osteoclast nuclei counts revealed approximately 2 - fold increases in average nuclei number in 2% O<sub>2</sub> versus 20% O<sub>2</sub>. Values are average nuclei per osteoclast +/- SEM, n = 4 discs.

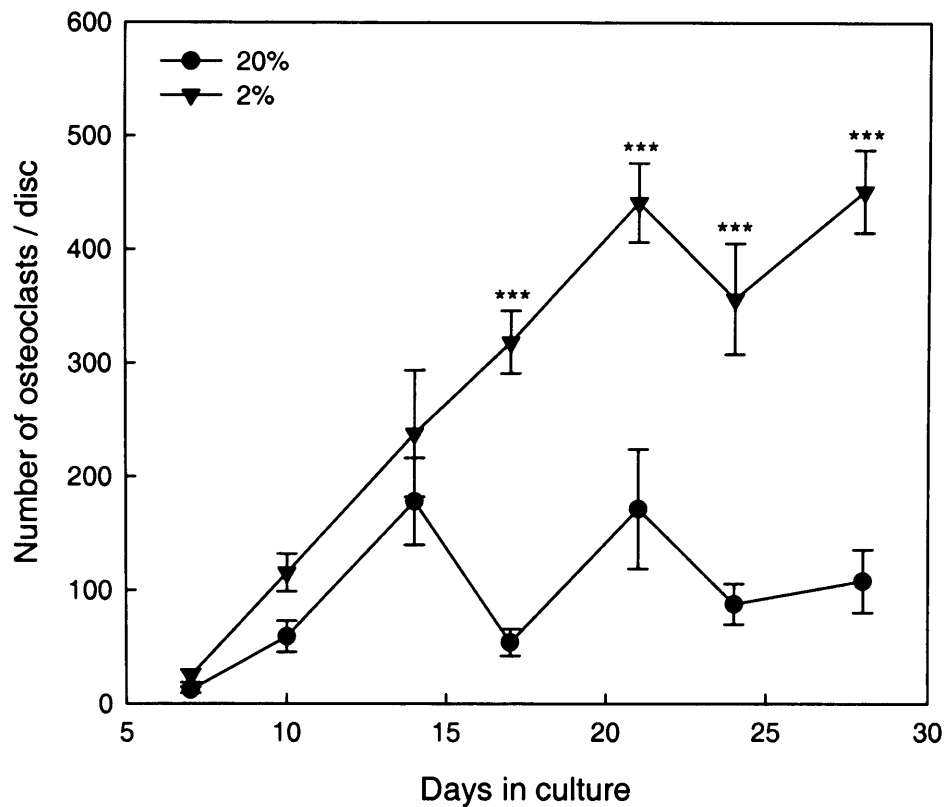


**Figure 3.3: Severe hypoxia stimulates osteoclastogenesis in human PBMC cultures.**

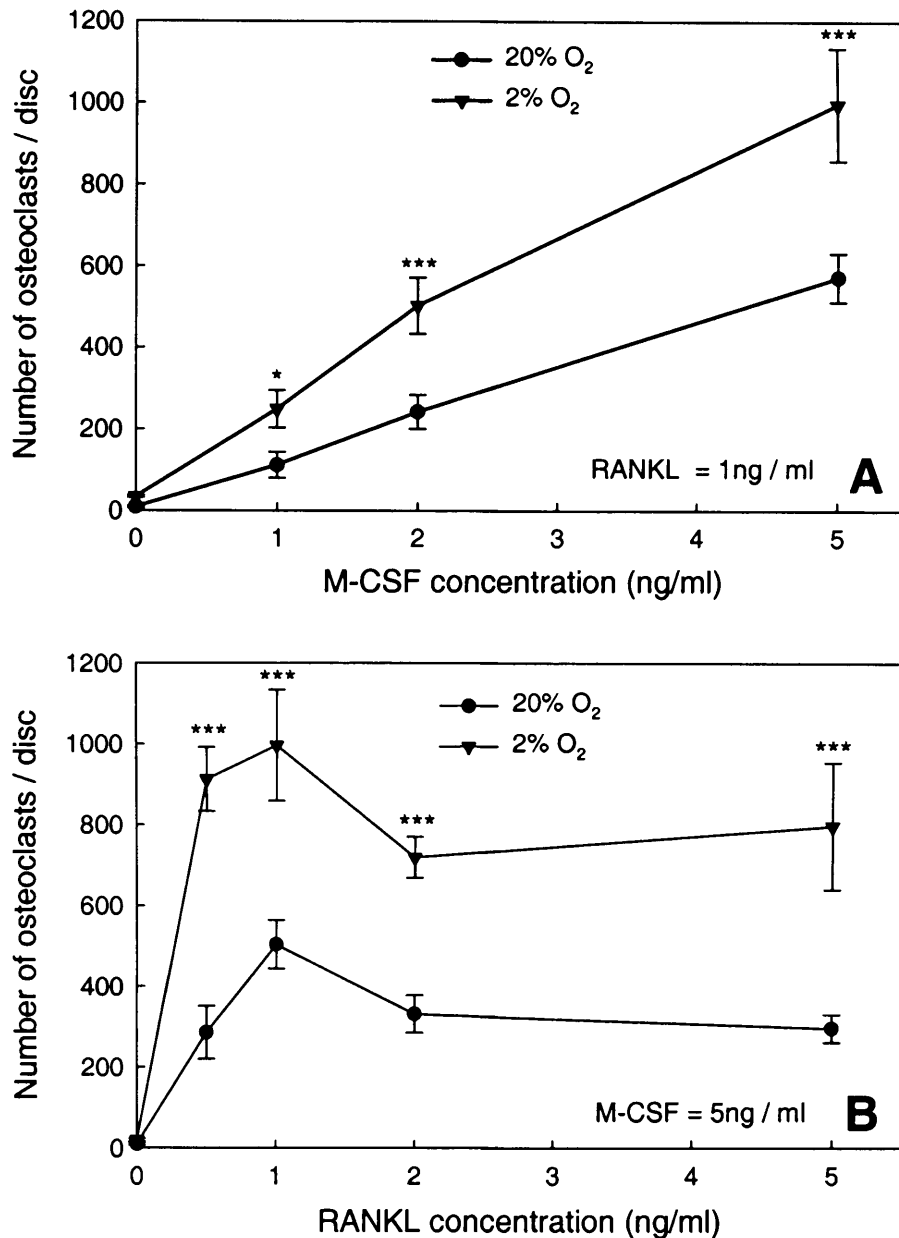
Maximum effects occur in 1-2% O<sub>2</sub> however significant stimulation of osteoclast formation is evident even in oxygen levels 1/100 that of atmospheric air. Resorption increases as oxygen tension decreases, with approximate 10-fold increases in 2% O<sub>2</sub> cultures compared to resorption in 20% O<sub>2</sub>. \* p < 0.05, \*\* p < 0.01 vs. 20% O<sub>2</sub> osteoclast number; # p < 0.05, ## p < 0.01 vs. 20% O<sub>2</sub> resorption. Values are means ± SEM; n = 8;



**Figure 3.4: Hypoxia does not stimulate human osteoclast activity in the absence of an acid stimulus.** Human osteoclast forming PBMNCs were cultured for 17 days in 20% O<sub>2</sub>; 14 days in 20% O<sub>2</sub> followed by 3 days in 2% O<sub>2</sub>; or 17 days in 2% O<sub>2</sub>. Medium pH was maintained above 7.43 (final values shown below bars) to limit acid-activation of osteoclasts. Initial exposure to 20% O<sub>2</sub> followed by treatment with 2% O<sub>2</sub> for the final 3 days of culture had no effect on osteoclast number or activity. Absence of an acid stimulus allowed only background resorption to occur in 2% O<sub>2</sub> treated cultures. \*\*\* p < 0.001 vs. 20% O<sub>2</sub> osteoclast number. Values are means  $\pm$  SEM; n = 8.



**Figure 3.5: Hypoxia increases the overall rate of osteoclast formation in long-term cultures.** Osteoclast formation was assessed every 3 – 4 days in cultures exposed to either 20% or 2% O<sub>2</sub>. Osteoclasts were first observed at day 7 of culture regardless of pO<sub>2</sub>. Osteoclast numbers in 2% O<sub>2</sub> increased to day 28 of culture, at this point osteoclast number was 4.5-fold increased in 2% O<sub>2</sub> relative to 20% O<sub>2</sub>. In contrast the rate of osteoclast formation in cultures exposed to 20% O<sub>2</sub> was decreased. Values are mean  $\pm$  SEM; n = 8.



**Figure 3.6: The hypoxia effect is dependent on permissive levels of M-CSF and RANKL.**

**A:** hPBMNC were cultured in the presence of 1ng/ml of RANKL and the M-CSF concentration varied. In the absence of M-CSF, minimal osteoclast formation occurred and resorption was absent; hypoxia (2% O<sub>2</sub>) failed to stimulate osteoclast formation. Osteoclast formation increased with M-CSF concentration; hypoxia elicited a ~2-fold stimulation. \* p < 0.05, \*\*\* p < 0.001 vs. respective 20% O<sub>2</sub> value. Values are mean ± SEM; n = 8.

**B:** hPBMNC were cultured in the presence of 5ng/ml M-CSF and the RANKL concentration varied. Osteoclast formation was dependent upon the presence of RANKL, regardless of pO<sub>2</sub>. Peak stimulation of osteoclastogenesis was observed in the presence of 1ng/ml RANKL in both 20% and 2% O<sub>2</sub>. Hypoxia was unable to stimulate osteoclast formation in the absence of RANKL; however, it increased the maximal number of osteoclasts capable of forming in response to RANKL stimulation. \*\*\* p < 0.001 vs. respective 20% O<sub>2</sub> value. Values are mean ± SEM; n = 8.

sensitive to M-CSF. When M-CSF was present at 5 ng/ml and RANKL was omitted from the medium, osteoclasts also failed to form. Again, this deficit could not be rectified by culturing in 2% O<sub>2</sub> (**Figure 3.6B**). Maximal stimulation of osteoclast formation was elicited by 1 ng/ml RANKL in both 20% and 2% O<sub>2</sub>.

### ***IL-6 production is increased in hypoxic cultures***

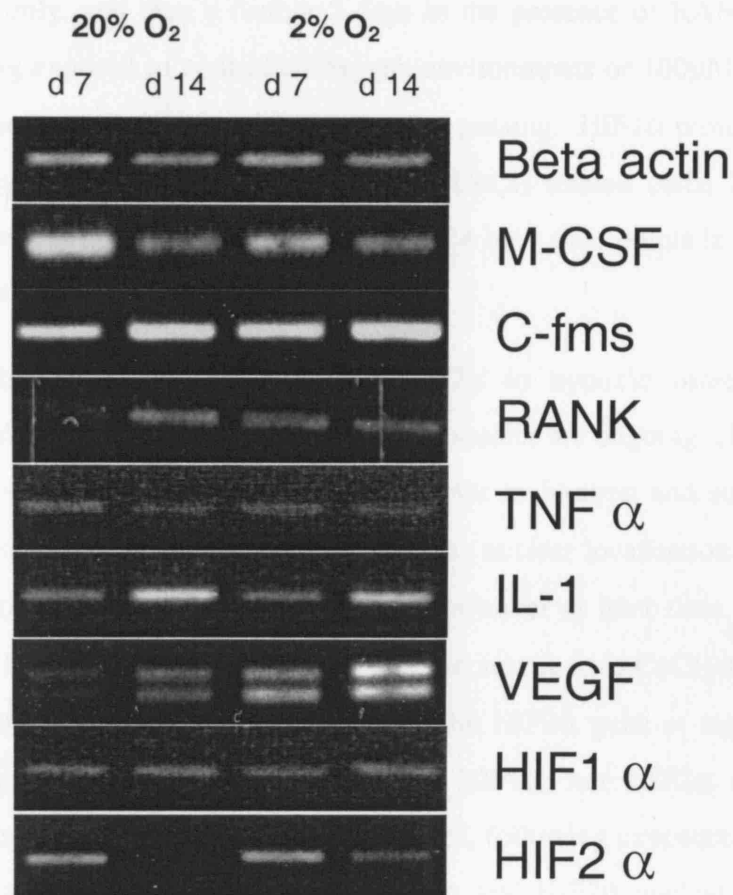
Analysis of IL-6 production by ELISA revealed 2 - fold increases in IL-6 protein levels in culture medium from cells grown in 2% O<sub>2</sub>, compared to those grown in 20% O<sub>2</sub> (133.3 pg/ml and 77.5 pg/ml, respectively after 7 days in culture; 271.4 pg/ml and 155.6 pg/ml, respectively after 14 days in culture). TNF $\alpha$  protein levels were below the level of detection of the assay (< 1.4 pg/ml) at all time points investigated, regardless of pO<sub>2</sub>.

### ***mRNA for VEGF is upregulated in hypoxic osteoclasts***

Osteoclasts formed in 20% and 2% O<sub>2</sub> were analysed for alterations in mRNA expression levels using RT-PCR (**Figure 3.7**). Consistent with the observation that M-CSF and RANKL were both required for osteoclastogenesis and the hypoxia response, RANKL mRNA could not be detected in human osteoclast cultures, and M-CSF mRNA expression did not correlate with increased osteoclast formation. The expression of the receptors for these cytokines, c-fms and RANK, showed no clear alterations in response to hypoxia.

The expression of mRNAs for key inflammatory and angiogenic cytokines was also investigated (**Figure 3.7**). VEGF isoforms 165 and 121 were easily detectable in human osteoclast cultures, and expression increased in cells formed in 2% O<sub>2</sub>. Expression of IL-1 $\alpha$ , IL-1 $\beta$ , TNF $\alpha$  and TGF $\beta$ <sub>1</sub> was also detected in human osteoclast cultures. None of these were obviously affected by hypoxia, although some changes were observed relative to time in culture. I was able to detect mRNA for both HIF1 $\alpha$  and HIF2 $\alpha$  in human osteoclasts regardless of pO<sub>2</sub>. HIF1 $\alpha$  expression was constitutive whereas HIF2 $\alpha$  expression varied depending on the time in culture.



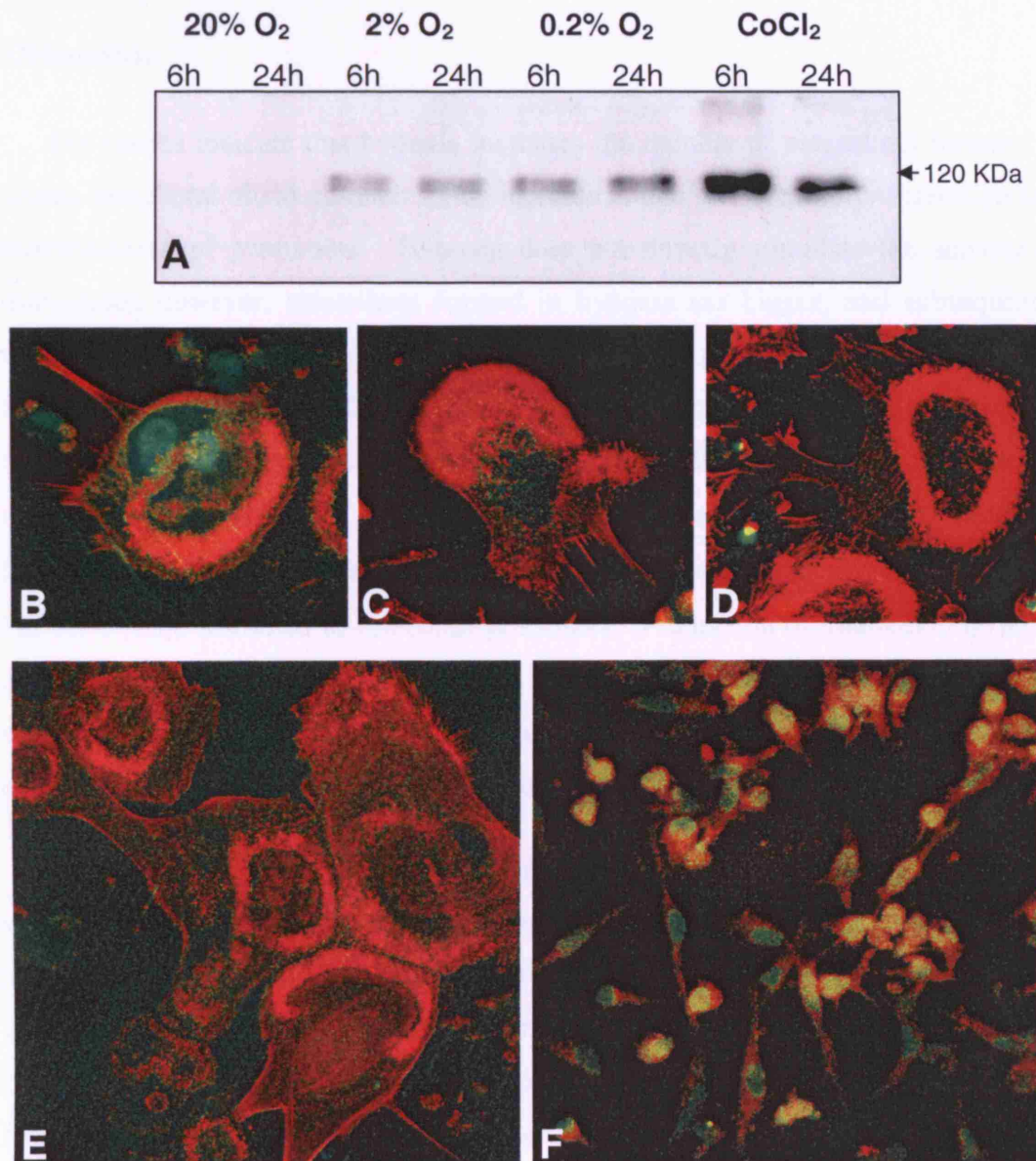


**Figure 3.7: Hypoxia upregulates mRNA for VEGF in osteoclast cultures.** cDNA derived from osteoclasts cultured for 7 and 14 days at 20% or 2% O<sub>2</sub> on dentine was analysed for the expression of a variety of osteoclast markers and osteoclastogenic cytokines, cell numbers were normalised by beta actin expression. VEGF was consistently strongly upregulated in hypoxic osteoclasts, whereas the changes in gene expression levels of other factors were only relative to stage of differentiation, not pO<sub>2</sub>. RANKL was not detectable in hPBMNC cultures (data not shown).

### ***Stabilisation of HIF $\alpha$ proteins in response to hypoxia***

Western blotting was used to investigate stabilisation and nuclear translocation of HIF1 $\alpha$  protein (**Figure 3.8A**). Osteoclasts were cultured for 4 days in the presence of M-CSF only, and then a further 2 days in the presence of RANKL and M-CSF, before being exposed to controlled oxygen environments or 100 $\mu$ M CoCl<sub>2</sub>. Nuclear extracts were prepared at 6 and 24 hours after gassing. HIF1 $\alpha$  protein was stabilised most strongly at the 6 hour time point in CoCl<sub>2</sub> treated cells; however, HIF1 $\alpha$  stabilisation was also observed in both 6 and 24 hour time points in 2% and 0.2% O<sub>2</sub> treated cells.

The stabilisation of both HIF1 $\alpha$  and HIF2 $\alpha$  in hypoxic osteoclasts was also investigated using immunofluorescence (these studies are ongoing) (**Figure 3.8B - F**). Treatment with 100 $\mu$ M CoCl<sub>2</sub> for 6 hours prior to fixation and staining led to the nuclear localisation of HIF1 $\alpha$  and HIF2 $\alpha$ . This nuclear localisation was consistently observed for HIF1 $\alpha$  throughout the culture, however at later time points in culture (day 14) HIF2 $\alpha$  nuclear localisation was lost in response to CoCl<sub>2</sub> stimulation. This correlates with the decreased expression of the HIF2 $\alpha$  gene at more differentiated stages of culture (see Figure. 3.8). Neither HIF1 $\alpha$ , nor HIF2 $\alpha$  stabilisation was observed using immunofluorescence techniques, following exposure of osteoclasts to 2% O<sub>2</sub> in 4, 7, 10 or 14 day cultures. HIF1 $\alpha$  and HIF2 $\alpha$  nuclear localisation was observed in osteoclasts exposed to 0.2% O<sub>2</sub> for 6 hours at day 4.



**Figure 3.8: HIF stabilisation in CoCl<sub>2</sub> and hypoxia treated human osteoclasts.**

**A.** Western blot analysis of osteoclast nuclear protein extracts revealed stabilisation of HIF1α at 6h and 24h post-gassing in 2% and 0.2% O<sub>2</sub> and CoCl<sub>2</sub> treated groups.

**B – D.** Osteoclast cultures on dentine were fixed and stained for the presence of HIF1α (rhodamine phalloidin, red: F-actin; FITC, green: HIF1α). **B.** Treatment with 100μM CoCl<sub>2</sub> induced the stabilisation of HIF1α in mature osteoclasts; note the strong nuclear staining. **C & D.** Normoxia (20% O<sub>2</sub>) and long-term hypoxia (2% O<sub>2</sub>) failed to induce nuclear localisation of HIF1α proteins.

**E & F.** Osteoclast cultures on dentine were fixed and stained for the presence of HIF2α (rhodamine phalloidin, red: F-actin; FITC, green: HIF1α). **E.** Mature osteoclasts (day 14), treated with 100μM CoCl<sub>2</sub> for 6 hours prior to staining, did not stabilise HIF2α. **F.** Short-term (6h) severe hypoxia (0.2% O<sub>2</sub>) induced stabilisation of HIF2α in day 4 cultures of hPBMNC (i.e., pre-osteoclast formation)

## Discussion

My results indicate that hypoxia increases the number of osteoclasts forming in human peripheral blood cultures. This increase is due to increased proliferation and differentiation of precursors. Hypoxia does not directly stimulate the activity of osteoclasts; however, osteoclasts formed in hypoxia are bigger, and subsequently resorb more dentine. The hypoxia response requires the presence of permissive levels of both M-CSF and RANKL, indicating that these factors are not being produced endogenously in the hypoxic cultures; however, PBMNC exposed to hypoxia are more sensitive to RANKL, and M-CSF. Osteoclasts express both HIF $\alpha$  subunits; HIF1 $\alpha$  is constitutively expressed throughout the lifespan of the osteoclast, whereas HIF2 $\alpha$  is more restricted to osteoclast precursors. Production of interleukin 6 (IL-6) and expression of the angiogenic cytokine vascular endothelial growth factor (VEGF) was increased in cultures exposed to hypoxia; no other osteoclastogenic factors were consistently identified as being upregulated in hypoxic cultures.

Throughout the course of my investigations a variety of method-related factors were found that profoundly influenced the ability of PBMNC to form osteoclasts *in vitro*. It was necessary to batch test foetal calf serum, as many batches were completely inhibitory to osteoclast formation, regardless of the potency of the cytokines. Variations in the source tusk of the dentine discs also affected osteoclast formation; bone chips were trialled as a substitute, but found to be even more unreliable. Preparations of RANKL and M-CSF from different batches and suppliers differ greatly in their ability to induce osteoclast formation. Batch testing was essential to ensure the osteoclastogenic activity of these factors. The sharp peak in osteoclast formation seen at 1 ng/ml of RANKL was characteristic of the recombinant protein expressed in *E. coli* (RnD Systems); increasing the concentration above this level inhibited osteoclast formation. RANKL preparations expressed in Chinese hamster ovary cells (Amgen) produced a much flatter dose-response curve with no inhibitory effect at high concentrations. Addition of high levels of M-CSF led to very high levels of osteoclast formation in 20% O<sub>2</sub>, such that no differences could be observed between hypoxia and normoxia. For this reason, concentrations of RANKL

and M-CSF were maintained at levels permissive for osteoclast formation in 20% O<sub>2</sub> cultures.

Hypoxia consistently stimulated osteoclast formation irrespective of the blood donor, although variations in the abilities of mononuclear cells to form osteoclasts were observed between donors. The level of osteoclast formation was also dependent on the state of health of the donor; for example, in subjects suffering from colds and similar infections, the total white cell count was higher but the number of osteoclasts forming was reduced.

The stimulation of human osteoclast formation from peripheral blood mononuclear cells by hypoxia was similar to that observed using mouse marrow. This is an important finding because it suggests that hypoxic stimulation of osteoclastogenesis is a general rather than species-specific phenomenon. Furthermore, the results from human cells suggest a direct effect of hypoxia on osteoclast precursors, as the human culture model system effectively removes the potential confounding factor of stromal cell interference. Even after 28 days, I failed to observe stromal/fibroblastic colonies in any of my cultures; moreover, all cells in the cultures stain TRAP positive (not a characteristic of stromal cells). Activated T lymphocytes also express RANKL and are capable of supporting osteoclastogenesis *in vitro* (Horwood *et al.*, 1999); however, they are non-adherent in culture, and in the present method, the dentine/bone discs are washed following sedimentation and attachment of cells to remove any T lymphocyte contamination (Massey *et. al.*, 2001). Furthermore, I failed to detect expression of RANKL by RT-PCR in osteoclast-forming hPBMC cultures, and osteoclast formation was dependent on the presence of exogenously provided RANKL. The dose response curves indicated that RANKL and M-CSF were not being endogenously produced in hypoxic or normoxic cultures; furthermore, the lack of osteoclasts in hypoxic cultures lacking M-CSF suggests that, in this system, VEGF induced by hypoxia is not capable of stimulating osteoclast formation in its stead, in contrast to the results reported by Niida *et. al.* (1999).

The osteoclasts formed in hypoxia were larger than those formed in normoxia, indicating a greater level of cell fusion. This effect was also related to the length of

time in culture; the longer cells were in culture for, the larger the osteoclasts became. Macrophages also possess the ability to fuse to form multinucleated giant cells associated with foreign bodies. The mechanism by which macrophages and osteoclasts fuse have not been clearly identified; however, it is thought to involve the CD44 molecule (Sterling *et al.*, 1998). A recent report indicates that the cell fusion event is associated with cleavage of intracellular domains of the CD44 molecule by presenilin (Cui *et al.*, 2005), deletion of presenilin leads to severe skeletal defects (Shen *et al.*, 1997).

Exposure of mature human osteoclasts to hypoxia over 3 days did not alter cell numbers, contrary to what was observed in the rat mature osteoclast model system. The lack of effect of transient hypoxia in human cultures is most likely due to the combined effect of decreased survival or attachment of osteoclasts and increased formation in hypoxia. It also revealed that hypoxia was incapable of stimulating the activity of osteoclasts; increased resorption could be accounted for by the increased size of the osteoclasts formed in low oxygen. However, it is important to note that, *in vivo*, hypoxia would cause an attendant acidosis due to increased anaerobic metabolism and reduced perfusion, which is sufficient to induce osteoclast activity (Arnett and Dempster, 1987; Arnett and Spowage, 1996). This effect was minimised within my culture system by the addition of excess medium, and measurement of pH at the termination of cultures indicated that medium pH was not affected by hypoxia.

Increased levels of IL-6 protein and VEGF expression were observed in hypoxic osteoclast cultures. Both of these cytokines are osteoclastogenic (Kudo *et al.*, 2003; Aldridge *et al.*, 2005); it is possible that they are at least partially responsible for the increased osteoclast formation observed in hypoxic cultures. Hypoxia in macrophages induces the expression of TNF $\alpha$  and IL-1 (Lewis *et al.*, 1999). I did not observe any changes in expression of either of these factors in hypoxic osteoclasts, and levels of TNF $\alpha$  protein were below the limits of detection in our assay system. This indicates that these two factors are unlikely to be involved in mediating the hypoxia effect.

Macrophages rapidly switch to anaerobic glycolysis when exposed to hypoxia; this is mediated by the HIF signalling pathway (Lewis *et al.*, 1999). Absence of

HIF1 $\alpha$  leads to a failure of neutrophil and macrophage formation and activity (Cramer *et al.*, 2003). Bone resorption is an energy-intensive process, and osteoclasts have a large number of mitochondria (Williams *et al.*, 1997). The relative extent to which osteoclasts can derive their energy from anaerobic glycolysis is not clear, however, my results clearly demonstrate that they can produce sufficient energy for bone resorption in hypoxia.

Human osteoclasts formed in hypoxia were present in cultures for longer than those formed in 20% O<sub>2</sub>. The increase numbers of osteoclasts present in long-term hypoxic cultures could be due to an extended life span or to increased precursor proliferation. HIF1 $\alpha$  and HIF2 $\alpha$  stabilisation has been linked with enhanced cell survival, HIF1 $\alpha$  expression was unchanged over the time course of cultures, however HIF2 $\alpha$  expression was diminished in 20% O<sub>2</sub> cultures at later time points. The response to long-term hypoxia is regulated to a greater extent by HIF2 $\alpha$  rather than HIF1 $\alpha$  (Uchida *et al.*, 2004). It is possible that variations in the survival response to hypoxia could be due to changes in HIF2 $\alpha$  expression possibly resulting in increased production of factors such as IGF1, which has been shown to increase survival of haematopoietic cells (Rodriguez-Tarduchy *et al.*, 1992).

The maintenance of low level HIF2 $\alpha$  expression at later stages of hypoxic cultures, compared to its absence in 20% O<sub>2</sub> cultures at day 14, could indicate that more cells were at an earlier stage of differentiation, or perhaps might give an indication of involvement of HIF2 $\alpha$  in the hypoxic response. HIF2 $\alpha$  has been identified in mature macrophages and it is possible that extended HIF2 $\alpha$  expression in hypoxic osteoclast cultures is a carryover from this differentiation pathway, i.e. more cells are existing in a precursor state; as they differentiate to osteoclasts they lose HIF2 $\alpha$  expression. It could also indicate macrophage contamination of the osteoclast population, as some subsets of macrophages express high levels of HIF2 $\alpha$  (Talks *et al.*, 2000).

The detection of HIF1 $\alpha$  and HIF2 $\alpha$  proteins in osteoclast cultures is difficult due to the transient nature of the proteins; the half-life of HIF1 $\alpha$  protein is less than 5 minutes in normoxia (Huang *et al.*, 1996). CoCl<sub>2</sub>, a hypoxia mimic that inhibits the

activity of the HIF prolyl hydroxylases, was able to stabilise both HIF1 $\alpha$  and HIF2 $\alpha$  in osteoclast precursors, and HIF1 $\alpha$  in mature osteoclasts. Although CoCl<sub>2</sub> mimics hypoxia in the short-term, long-term exposure leads to cell death. HIF2 $\alpha$  stabilisation was not observed in mature osteoclasts in response to CoCl<sub>2</sub> treatment, consistent with its reduced mRNA expression at later time periods of culture. Hypoxia (2% and 0.2% O<sub>2</sub>) stabilised HIF1 $\alpha$  protein in osteoclast precursors, as determined by Western blotting; however, only 0.2% O<sub>2</sub> stabilised HIF1 $\alpha$  and HIF2 $\alpha$  when determined using immunofluorescence. It is possible that hypoxia is stabilising HIF in these osteoclast cultures but the time taken to process the samples was sufficient to degrade the HIF protein before fixation. Dedicated hypoxia workstations are used to counter this problem in some laboratories. Furthermore, commercial antibodies against the HIF proteins have proved unreliable; I am currently testing several other antibodies.

Osteoclast responses to hypoxia could conceivably be mediated via mechanisms that do not involve HIF. Alterations in calcium signalling caused by hypoxia-induced ion channel activity activate calcium-regulated pathways in other cell types (Seta *et al.*, 2004). Oscillations in intracellular calcium levels activate the transcription factor NFATc1 in osteoclasts, which induces osteoclastogenesis (Takayanagi *et al.*, 2002). Although ROS production is controversial during hypoxia (reviewed in Kietzmann and Gorlach, 2005), the method that we use to produce a hypoxic environment would mean that the cells could experience short periods of reoxygenation during medium changes. Hypoxia/reoxygenation has been shown to cause ROS production through complex III of the mitochondrial electron transport chain in endothelial cells (Therade-Matharan *et al.*, 2004). Hydrogen peroxide and oxygen radicals enhance Ca<sup>2+</sup> release in organ culture (Fraser *et al.*, 1996; Garrett *et al.*, 1990), and have been implicated in the bone loss associated with oestrogen deficiency (Lean *et al.*, 2005). Moreover, Lean and colleagues (2005) have recently shown that hydrogen peroxide can stimulate osteoclast formation directly. ROS are used as second messengers in the activation of NF $\kappa$ B (Schreck *et al.*, 1991), and antioxidants reduce NF $\kappa$ B activation in osteoclasts (Kim *et al.*, 2005). The effects of ROS on the activity of mature osteoclasts have been difficult to demonstrate (T.R. Arnett, personal communication), and I see no effect of hypoxia on osteoclast activity. I attempted to address this potential ROS production using two approaches. Firstly, by using N-



acetyl cysteine (30mM) as described by Lean et al (2005); secondly (and as described in Chapter 2) by adding ascorbic acid (50 and 100 µg/ml); however, addition of either of these factors to human osteoclast cultures led to extensive cell death regardless of pO<sub>2</sub> (data not presented).

It has recently been reported that the vitronectin receptor (VNR), αvβ3, is upregulated by hypoxia, through the action of the hypoxia inducible factor, in trophoblasts and melanoma cells (Cowden Dahl *et al.*, 2005). Osteoclasts utilise VNR for attachment to the bony surface (Horton *et al.*, 1991). Upregulation of αvβ3 by hypoxia in osteoclast precursors could help account for the increased osteoclastogenesis observed. Our laboratory experience shows that successful osteoclast formation in vitro is dependent on a relatively high initial seeding density of precursor cells; increased attachment to substrate would lead to closer contact between cells and may enhance osteoclast formation. I visualised VNR expression on mature osteoclasts in hypoxic and normoxic cultures using immunofluorescence; however, the expression levels were too high in both cases for any differences to be detected. Further investigation may require a Western blotting approach.

Recent work by Fukuoka and colleagues (2005) has shown that expression of IGF2, an osteoclastogenic factor, is increased in murine osteoclast cultures exposed to hypoxia; this IGF2 was produced by stromal cells present in the marrow cultures. However; in the human osteoclast culture system described here, few, if any stromal cells are likely to be present, suggesting that the osteoclastogenic action of hypoxia occurs directly on cells of the myeloid lineage.

The results presented here show very strong effects of alterations in pO<sub>2</sub> on osteoclast formation, and thus mineralised tissue resorption. They also demonstrate that this effect is probably independent of stromal cell participation, suggesting that hypoxia acts directly on osteoclast precursors. *In vivo*, however, the action of hypoxia will also involve effects on osteoblasts and other cells of mesenchymal origin.

# CHAPTER 4

## *Hypoxia inhibits bone formation by rat calvarial osteoblasts*

### **Introduction**

Osteoblasts produce the organic and mineral matrix that is the main component of bone. The combination of these factors gives bone its unique mechanical properties. Alterations in the properties of either of these components lead to changes in the structural integrity and consequently the ability of the bone to withstand the forces applied to it. Bone formation requires several discrete steps, the proliferation and differentiation of mesenchymal stem cells to osteoblast precursors, the production of organic collagenous matrix, and then the mineralisation of this organic matrix (Aubin *et al.*, 1995).

The ability of cultured primary osteoblastic cells to form bone nodules *in vitro* allows the investigation of many aspects of this differentiation and matrix deposition pathway. Rodent models are very useful in this respect because they provide a readily accessible source of young, vigorous cells. The most commonly used technique is the isolation of osteoblasts by sequential digestion of neonatal rat or mouse calvaria. This method first uses treatment with trypsin to remove soft tissue, and then collagenase to strip the outer layers of the fibrous periosteum to release osteoblasts and their precursors (Wong and Cohn, 1975; Bellows *et al.*, 1986). These released cells are capable of differentiating into mature, functional osteoblasts that produce collagenous matrix nodules that mineralise when supplemented with a source of phosphate and ascorbate; the number of nodules formed is dependent upon addition of the synthetic glucocorticoid dexamethasone to the cultures (Bellows *et al.*, 1986). The bone produced by these cells has the histochemical appearance of woven bone (Bellows *et al.*, 1986). The population of cells released from calvaria is

heterogeneous, and is also capable of differentiating into chondrocytes, adipocytes and muscle cells (Grigoriadis *et al.*, 1988). The temporal expression of markers in osteoblast cultures indicates differentiation from a proliferating multipotential precursors to pre-osteoblasts, to mature osteoblasts and, finally to bone lining cells or osteocytes. Markers sequentially expressed include Runx2 expression at the pre-osteoblast stage (Ducy *et al.*, 1997); type 1 collagen at the pre-osteoblast and mature osteoblast stages; and osteocalcin and high alkaline phosphatase activity at the mature osteoblast stage (Aubin *et al.*, 1995).

Osteoblasts can also be derived from the bone marrow. One major drawback with using bone marrow cells is that the bone marrow contains both stromal and haematopoietic cells, which reduces the proportion of osteogenic precursors and increases the variability of the system (Herbertson and Aubin, 1997). Several cell lines are also available that have been shown to differentiate into osteoblasts and produce mineralised matrix. However, many aspects of osteoblast differentiation such as proliferation arrest and marker expression are altered in these cells (reviewed in Kartsogiannis and Ng, 2004). For this reason transformed cell lines are not utilised in our laboratory; normal, primary rat osteoblasts from calvaria perform the ultimate function of osteoblasts - they produce bone.

The effects of hypoxia on osteoblasts are of interest because the vasculature plays such an important role in bone formation during endochondral ossification. Bone formation cannot occur until the cartilage anlagen have been invaded by vessels that supply nutrients and oxygen, as well as removing cellular waste products. The stimulus for the angiogenic signal is still unknown, although the effector molecules include vascular endothelial growth factor (VEGF) (Zelzer *et al.*, 2002). One of the most potent stimuli for the production of angiogenic factors is hypoxia. Furthermore, collagen production is dependent on the availability of oxygen for the enzymatic modifications required for its stability and cross-linking (Counts *et al.*, 1978; Kagan and Li, 2003; Kikuchi *et al.*, 1983).

Previous studies on the effects of hypoxia on osteoblasts have all been performed for transient time periods; the long-term effects of hypoxia have not been examined. Furthermore, the vital function of osteoblasts, bone formation, has been relatively

neglected as an endpoint of these studies. Hence I investigated the effect of chronic hypoxia on bone formation by rat calvarial osteoblasts and how hypoxia affected gene expression and collagen deposition in these primary cells.

## Materials and methods

### *Reagents*

Culture medium and buffers were purchased from Gibco (Paisley, UK). Cylinders containing custom mixtures of O<sub>2</sub>, CO<sub>2</sub> and N<sub>2</sub> were purchased from BOC Gases (London, UK). All other reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

### *Bone nodule formation assay*

Primary rat osteoblastic cells were obtained by sequential enzyme digestion of excised calvarial bones from two-day-old neonatal Sprague-Dawley rats using a three-step process (0.25% trypsin for 10 min, 0.2% collagenase type II for 30 min, and 0.2% collagenase type II for 60 min at 37°C), rejecting the first two digests (Bellows *et al.*, 1986). The cells were washed and plated into a T-75 flask in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal calf serum / 2mM L-glutamine / 100U/ml penicillin / 100µg/ml streptomycin / 0.25µg/ml amphotericin (from here on referred to as DMEM). When confluent, cells were removed from the flask using 0.25% trypsin, washed and counted before being placed into 24 well trays containing 1cm diameter Melinex discs (Dupont Teijin Films, Dumfries, UK) at a density of  $5 \times 10^4$  cells / well in DMEM. After 24 hours discs bearing cells were transferred to 25 cm<sup>2</sup> flasks with polyethylene 'plug-seal' caps (Falcon, Becton Dickinson, Oxford, UK), containing 8 ml of DMEM supplemented with 50µg/ml ascorbate /  $10^{-8}$ M dexamethasone / 2mM β-glycerophosphate (six discs per flask). This concentration of phosphate is used because excessive levels cause ectopic mineralisation (Chung *et al.*, 1992). The flasks were then flushed for 2 min with gas mixtures containing 5% CO<sub>2</sub> and 20, 12, 5, 2, 1, or 0.2% O<sub>2</sub> (balance N<sub>2</sub>) as described previously. The sealed flasks were incubated at 37°C in a standard incubator containing 5% CO<sub>2</sub> / 95% atmospheric air and re-gassed daily. Medium was changed every 3 - 4 days. Bone nodules were visualised by staining with alizarin red S (Sigma, Poole, UK) to detect calcium. Briefly, discs in flasks were washed with PBS and fixed for 3 minutes in 2.5% glutaraldehyde. Discs were washed three times in 70% ethanol and left to dry inverted for 30 minutes. 1% w / v alizarin red S in

distilled water was then added to flasks for 3-5 minutes and discs were washed three times in 50% ethanol and dried before quantification of nodule area. Nodule area was determined by imaging discs at 800 dpi using a high-resolution flatbed scanner (Epson Perfection Photo 3200, Epson, Hemel Hempstead, UK). Colour information was removed and binary images analysed using Scion Image analysis software (Scion Corporation, Frederick, MD, USA; <http://www.scioncorp.com/>) at constant threshold level and minimum particle size, to determine the plan surface area of mineralised bone nodules (Hoebertz *et al.*, 2002).

### ***Proliferation assay***

Primary osteoblasts were prepared as described above and cultured directly into 24 well trays. Trays were placed into gas tight fuse boxes which were flushed for 2 minutes with gas mixtures containing 5% CO<sub>2</sub> and 20 or 2% O<sub>2</sub> (balance N<sub>2</sub>) through holes drilled in the lid. Cell proliferation was determined as <sup>3</sup>H-thymidine incorporation into deoxyribonucleic acid (DNA), essentially as described previously (Gray *et al.*, 1992). Calvarial osteoblasts were labelled with 1 µCi/mL of [6-<sup>3</sup>H] thymidine (22.0 Ci/mmol; 814 GBq/mmol) for the final 6 hours of culture. Cell layers were washed three times with phosphate-buffered saline (PBS) containing 1 mM unlabeled thymidine, and then detached using 0.25% trypsin for 5 minutes at 37°C, and precipitated with 7.5% trichloroacetic acid (TCA) in the presence of 0.2% bovine serum albumin (BSA), followed by centrifugation at 1500g. Pellets were washed with 7.5% TCA/0.2% BSA, recentrifuged, and then digested overnight at 37°C in 0.2 M NaOH and mixed with scintillation cocktail (Wallac Optiphase Hisafe II, Fisher Scientific, Loughborough, UK) for scintillation counting.

### ***Apoptosis assay***

Osteoblast apoptosis was assessed using a kit (Cell Death Detection ELISA<sup>PLUS</sup>, Roche Diagnostics Ltd, Lewes, East Sussex, UK) according to the manufacturer's protocol. This assay measures cytoplasmic histone DNA fragments (mono- and oligonucleosomes) produced after cell death by using mouse monoclonal antibodies against histones and DNA. Briefly, cells were lysed in situ after being cultured in supplemented medium, for 6, 12, and 18 days in 20% or 2% O<sub>2</sub>. Mono- and

oligonucleosomes released into the cytoplasm were detected by measuring the absorbance at 450 and 490 nm; duplicate determinations were made. The results were normalized to osteoblast numbers in parallel wells, assessed using a haemocytometer, after sequential treatment with 0.2% collagenase type II for 30 min, and 0.25 % trypsin for 10 min to release cells.

### ***Alkaline phosphatase assay***

Cells were grown in 24 well trays as described above. Monolayers were washed in PBS and then scraped into 500µl of distilled water. 2 wells were combined and the cell suspension transferred into a 15ml tube and sonicated in ice for 10 minutes. Cell debris was removed by centrifugation at 500g for 10 minutes at 4°C. Supernatant was used to determine alkaline phosphatase activity and total protein. Alkaline phosphatase activity was determined using an assay from Biotran Diagnostics (CA, USA) according to the manufacturers instructions. Alkaline phosphatase activity was normalized to total cell protein content by measuring total protein using the Lowry protein assay (P-5656, Sigma, Poole, UK) according to the manufacturers instructions. Alkaline phosphatase activity is expressed as U/mg of total protein. Alkaline phosphatase was also visualized within rat calvarial osteoblast monolayers using the Alkaline Phosphatase Kit (86C, Sigma, Poole, UK) according to the manufacturers instructions.

### ***Transmission electron microscopy***

Primary osteoblasts were prepared on Melinex discs as described above and cultured for 35 days in supplemented DMEM until abundant bone formation was observed. Discs were removed from flasks into 24 well trays and fixed in 2.5% glutaraldehyde and decalcified by overnight immersion in 1.9% glutaraldehyde 0.15M EDTA in phosphate buffered saline (PBS) at 4°C. Discs were rinsed in 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide and stained with 1% uranyl acetate (BDH) before being dehydrated through a graded ethanol series. Ethanol was replaced with resin through a graded series of ethanol: resin mixtures. The resin consisted of Agar 100, dodecenyl succinic anhydride, methyl nadic anhydride, mixed with N-benzyltrimethylamine as an accelerant; this mixture was put

under a dessicator and vacuum to remove gases. Samples were placed in fresh resin in embedding moulds and cured in an oven at 60°C for 48 hours. Adjacent thin sections (80 to 90 nm) were cut using a diamond knife (Diatome) on a Reichert Ultracut E ultramicrotome. The sections were collected on 300 mesh grids, counterstained with lead citrate, and viewed in a JEOL 1010 electron microscope.

#### ***Collagen analysis (performed by Dr Simon Robins)***

Melinex discs with their accumulated collagenous matrix were incubated with 0.5M acetic acid, pH 2.0, containing pepsin (1 mg/ml; Sigma, Poole, UK) at 4°C for 24 hours with gentle agitation. Insoluble material was removed by centrifugation and the residue was extracted similarly with pepsin for a further 24 hours. Collagenous proteins in the combined supernatant solutions were precipitated with NaCl at a final concentration of 1.2M and collected by centrifugation. Both pepsin-solubilised, precipitated collagen and the insoluble matrix fraction were separately hydrolysed in 6M-HCl at 107°C for 22 hours, after which time the acid was removed by evaporation. Hydroxylation of proline and lysine was assessed by quantifying Hyp, Pro, Hyl and Lys using an amino acid analyser (Waters Pico-Tag™ instrument, Manchester, UK). Total collagen was calculated assuming 300 moles Hyp per mole collagen.

#### ***Reverse transcriptase polymerase chain reaction (RT-PCR)***

RNA for RT-PCR was isolated from primary osteoblasts grown on melinex discs in gassed 25cm<sup>2</sup> tissue culture flasks. Monolayers were washed briefly in PBS and 1ml of TRIzol reagent (Invitrogen, Paisley, UK) was added to each flask and processed according to the manufacturers instructions. RNA pellet was dissolved in 50 µl of DEPC-treated water and 4 µl of total RNA was utilized for cDNA production using the ImPromII Reverse Transcription System (Promega, Southampton, UK) according to the manufacturers instructions. cDNA levels were normalized to beta-actin expression and used in polymerase chain reactions using primer pairs listed in **Table 4.1**.



<b>Gene product</b>	<b>Forward primer 5'-3'</b>	<b>Reverse primer 5'-3'</b>
<b>Beta actin</b>	GTT CGC CAT GGA TGA CGA T	TCT GGG TCA TCT TTT CAC GG
<b>Alkaline phosphatase</b>	CTC ATT TGT GCC AGA GAA	GTT GTA CGT CTT GGA GAC
<b>Osteocalcin</b>	GCA GAC ACC ATG AGG ACC CT	GCA GCT GTG CCG TCC ATA C
<b>TGF<math>\beta_1</math></b>	CGC AAC AAC GCA ATC TAT G	CAT GTC ATG GAT GGT GCC
<b>TGF<math>\beta_2</math></b>	GCA GAG TTC AGG GTC TTT CG	CAC CAC TGG CAT ATG TGG AG
<b>TGF<math>\beta_3</math></b>	TTG CGG AGA GAG TCC AAC TT	CAC ACA GCA GTT CTC CTC CA
<b>PLOD1</b>	CAA GCT GCA GGT GAA CTA C	CCA CAC TGA AGT AGT AGG TGC
<b>PLOD2</b>	CAG TAG ATG TCT ATC CGA	CAG AAC GAG CAT AGT ATC
<b>PLOD3</b>	GAG CCT TAG ATG AAG TGG	TCG TGG TAC ACC TCG TTG
<b>Collagen 1A1</b>	GCG AAG AAG ACA TCC CTG	CTG TCC AGG GAT GCC ATC
<b>VEGF</b>	GAG ACC CTG GTG GAC ATC T	CAC CGC CTT GGC TTG TCA C

**Table 4.1: Primer pairs used for amplification of osteoblast cDNA.**

### ***Chick limb-bud micromass culture***

Limb buds were harvested from chick embryos at Hamburger Hamilton stage 22 – 24 (Hamburger and Hamilton, 1951). Mesenchymal cells were released from the ectoderm by treatment with 2.5% trypsin for 10 minutes on ice. Cells were pelleted and resuspended in 50:50 Hams F12 / DMEM supplemented with 10% FCS. 20µl droplets containing  $2 \times 10^5$  cells were placed in 24 well trays and left to adhere for 2 hours at 37°C / 5% CO<sub>2</sub>. Cells were gently overlaid with medium and incubated for 3 days at 37°C/5% CO<sub>2</sub> in gas tight fuse boxes purged with gas mixtures as described previously. Cultures were stopped by addition of 2.5% glutaraldehyde and stained for cartilage with alcian blue. Cartilage nodule area was determined by imaging discs at 800 dpi using a high-resolution flatbed scanner (Epson Perfection Photo 3200, Epson, Hemel Hempstead, UK). Colour information was removed and binary images analysed using Scion Image analysis software (Scion Corporation, Frederick, MD, USA; <http://www.scioncorp.com/>) at constant threshold level and minimum particle size, to determine the plan surface area of cartilaginous nodules.

### ***Alcian blue staining***

The presence of cartilage in rat calvarial osteoblast cultures and chick limb-bud cultures was determined by staining for glycosaminoglycans using alcian blue. Cells grown on Melinex discs or in 24 well trays were fixed in 2.5% glutaraldehyde then washed in distilled water and stained with 1% w/v alcian blue in acetic acid for 30 minutes at room temperature. Cells were then thoroughly washed in distilled water prior to visualisation of proteoglycan-containing nodules.

### ***Statistical Analysis***

Statistical comparisons were made by one-way analysis of variance (ANOVA) using “Instat” software (version 1.3, Graph Pad, Inc, San Diego, CA); representative data are presented as means ± standard error of the mean (SEM). Significance was assumed at  $P < 0.05$ . Bonferroni adjustment was made for repeated comparisons. Results are presented for representative experiments that were repeated at least 3 times.

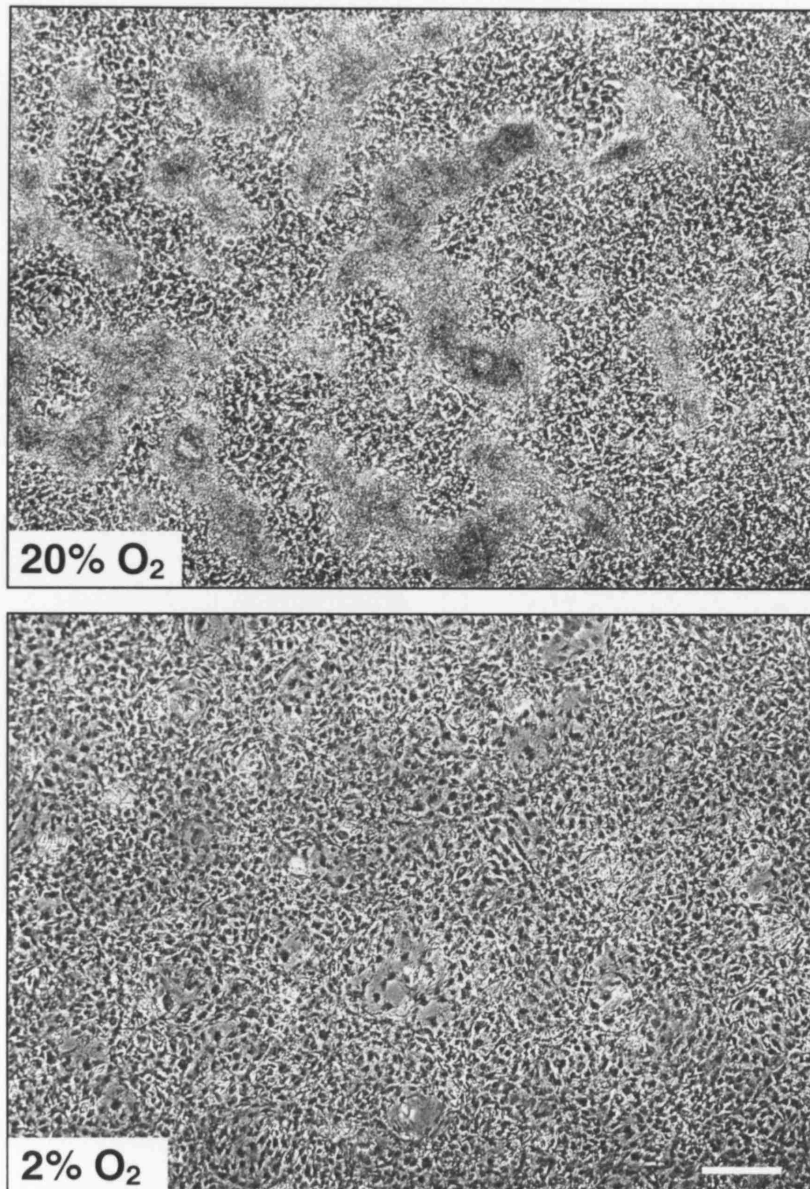
## Results

### *Inhibition of bone nodule formation by hypoxia*

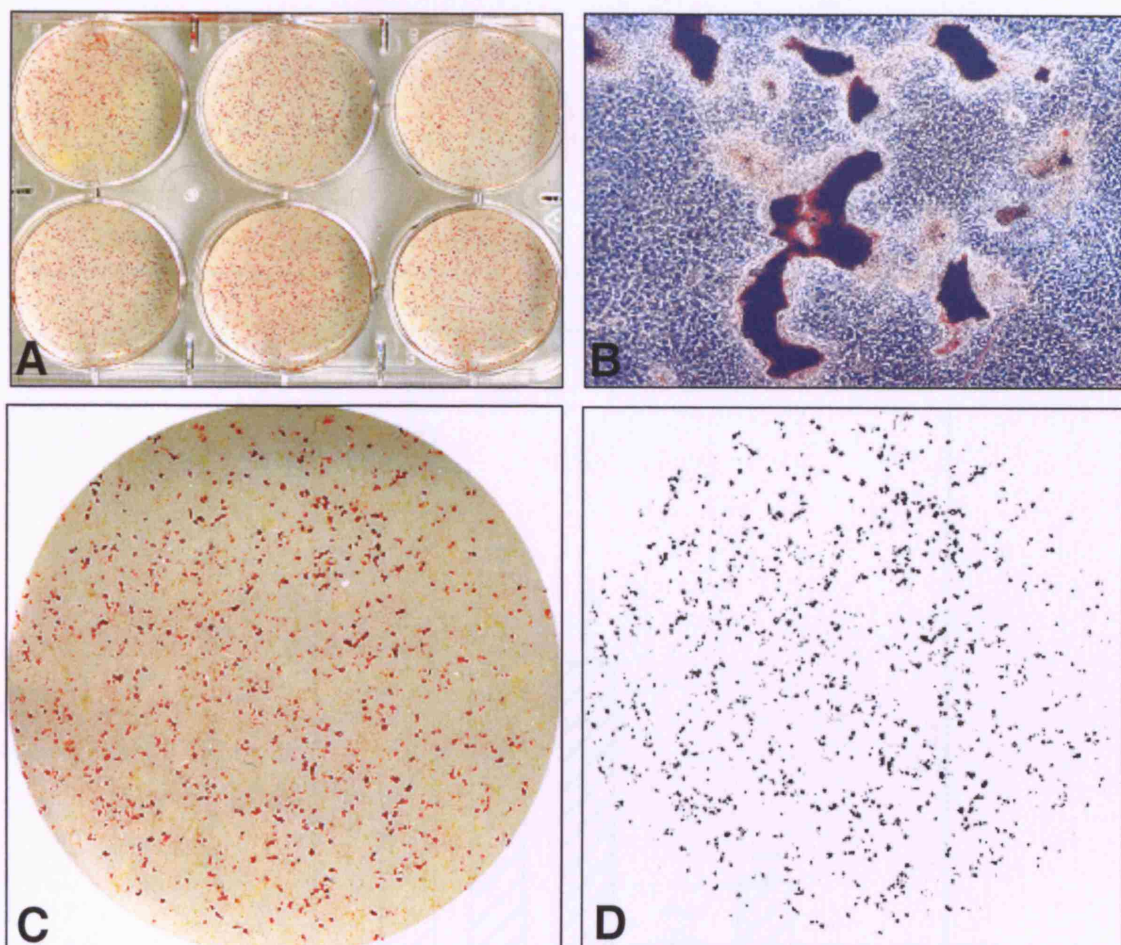
Abundant bone nodule formation occurred on Melinex discs bearing osteoblasts cultured in 20% oxygen for 18 to 24 days (**Figure 4.1**). Scanned images of bone nodules were obtained and processed as shown in **Figure 4.2**; area was quantified using image analysis software. Bone nodule formation decreased as  $pO_2$  was reduced (**Figures 4.1 & 4.3**). When  $pO_2$  was reduced to 12% or 5%, slight reductions in bone nodule area were seen; as  $pO_2$  was reduced further to 2%  $O_2$  and 1%  $O_2$  (moderate hypoxia) bone formation decreased 10-fold until in extreme hypoxia (0.2%  $O_2$ ) bone nodule formation was almost completely abolished (**Figure 4.3**). When the hypoxia mimic  $CoCl_2$  was added to osteoblast cultures at 100 $\mu$ M or 50 $\mu$ M concentrations, extensive cell death was observed when cultures were terminated; when  $CoCl_2$  concentration was reduced to 10 $\mu$ M, osteoblasts survived but no nodules were formed. Since hypoxia can cause increased acidification of cell cultures, experiments were performed using large volumes of culture medium to provide excess buffering capacity; medium was monitored regularly to ensure that operating pH (typically close to 7.40 before medium changes) did not differ significantly between cultures exposed to different  $O_2$  tensions.

To determine whether osteoblast function was most sensitive to the inhibitory effect of hypoxia at earlier or later stages of differentiation, osteoblasts were cultured in either 20% or 2%  $O_2$  for 6-day periods for a total of 18 days (**Figure 4.4**). Exposure to hypoxia (2%  $O_2$ ) for the first 6 days of culture followed by 20%  $O_2$  for the final 12 days resulted in a 3-fold decrease in bone nodule formation. Exposure to 20%  $O_2$  for the initial 6 days of culture and 2%  $O_2$  for the final 12 days of culture led to a 2-fold decrease in bone nodule formation. The cumulative area of nodule formation in these cultures approached that of cultures held at 20%  $O_2$  continuously, indicating that osteoblasts are able to recover from hypoxic insult.

The effect of hypoxia on mature osteoblasts was investigated by culturing osteoblasts to maturity at 20%  $O_2$  and then transferring them into reduced  $pO_2$ ; nodule formation was beginning at day 12 of culture when cells were transferred to



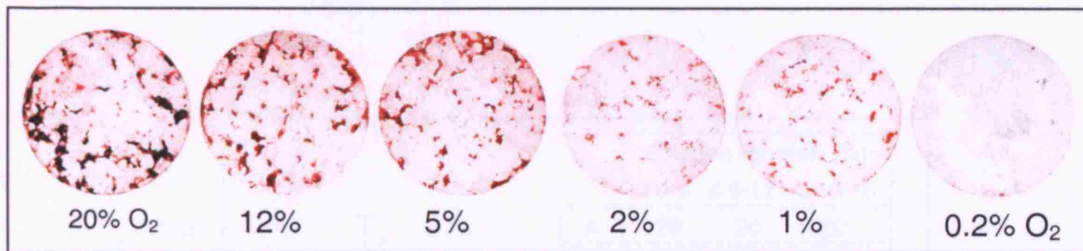
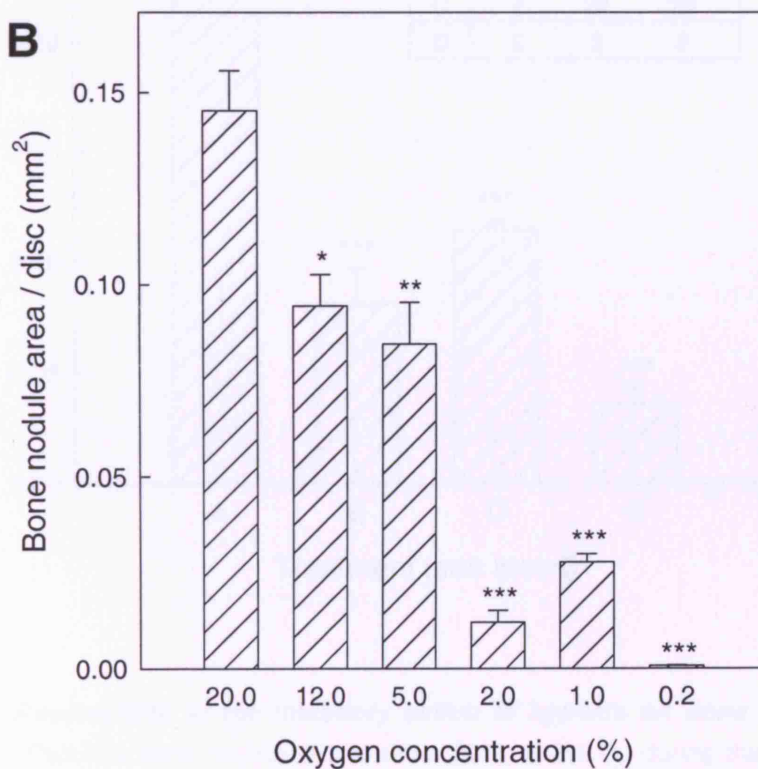
**Figure 4.1: Hypoxia inhibits osteoblast growth and bone nodule formation by osteoblasts.** Phase contrast micrographs of unstained rat calvarial osteoblasts seeded at an initial density of  $5 \times 10^4$  cells per well and cultured for 9 days in the presence of 50 $\mu$ g/ml ascorbate and 2 mM  $\beta$ -glycerophosphate. In 'normoxic' cultures (top), widespread, early bone formation (grey, 'trabecular' features) is evident; however, in hypoxic cultures (bottom), cell numbers are decreased and bone formation is absent. Scale bar = 200  $\mu$ m.



**Figure 4.2: Quantification of alizarin red-stained bone nodules.**

- A:** Low power image of a 6 well tray containing bone nodules stained with alizarin red.
- B:** High power image of alizarin red stained bone nodules (Original magnification 100x).
- C:** Scanned image of a single well of a 6 well tray containing bone nodules prior to removal of colour information.
- D:** Processed image of well shown in (C); colour information has been removed and image thresholded to produce a black and white image of nodules, which was quantified using image analysis software.

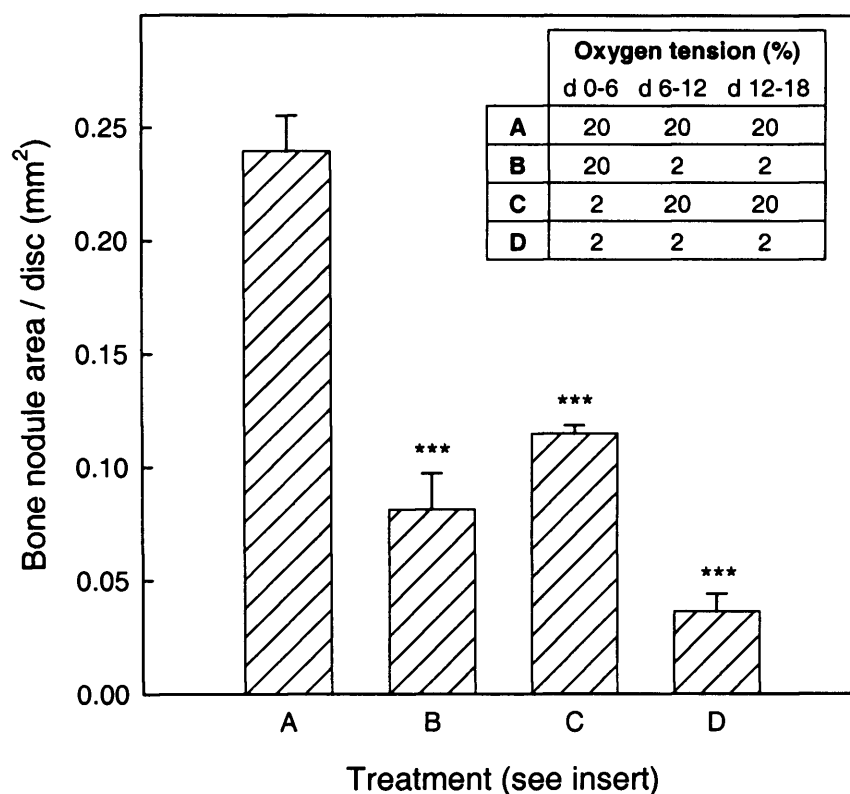


**A****B**

**Figure 4.3: Progressive inhibition of mineralised bone nodule formation by osteoblasts with decreasing O<sub>2</sub>.**

**A:** Representative low-power images of alizarin red-stained bone nodules formed by rat calvarial osteoblasts cultured for 18 days on 1 cm diameter Melinex discs in sealed tissue culture flasks gassed with 20% - 0.2% O<sub>2</sub> / 5% CO<sub>2</sub> / balance N<sub>2</sub>.

**B:** Bone nodule formation, quantified using image-analysis software, was slightly inhibited in 12% or 5% O<sub>2</sub>, strongly inhibited in 2% or 1% O<sub>2</sub> (hypoxia) and effectively abolished in 0.2% O<sub>2</sub> (severe hypoxia / ischaemia). \*  $p < 0.5$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  vs. 20% O<sub>2</sub>. Values are means  $\pm$  SEM;  $n = 6$



**Figure 4.4: Reversibility of the inhibitory action of hypoxia on bone formation by osteoblasts.** Cultures were maintained in either 20% or 2% O<sub>2</sub> during the time periods indicated in the key. Exposure to 2% O<sub>2</sub> for the maturation and matrix production stages (days 6 to 18; treatment group B) led to 3-fold reductions in bone nodule area. Exposure to 2% O<sub>2</sub> for the initial proliferation stage (days 0 to 6; treatment group C) resulted in 2-fold reductions in nodule area. The cumulative level of bone nodule formation in treatment groups B and C together approached that observed when osteoblasts were cultured continuously at 20% O<sub>2</sub> (treatment group A). \* p < 0.5, \*\* p < 0.01, and \*\*\* p < 0.001 vs. treatment group A (20% O<sub>2</sub>). Values are means ± SEM; n = 6.

altered  $pO_2$ , cultures were terminated at day 18. Decreasing  $pO_2$  to 12% led to approximately 50% reductions in nodule formation over cultures maintained in 20%  $O_2$  (**Figure 4.5**). Levels of nodule formation by osteoblasts transferred to 5%  $O_2$  or less were not significantly increased from the control culture stopped at day 12, i.e. from the time of transfer no mineralised matrix had been deposited.

### ***Inhibition of osteoblast proliferation and alkaline phosphatase activity by hypoxia***

The proliferation assay was performed at days 6, 12 and 18; at all time points culture in 2%  $O_2$  caused ~3-fold decreases in osteoblast proliferation, compared with 20%  $O_2$  (**Figure 4.6A**). Levels of osteoblast apoptosis, detected as nucleosome enrichment in cell monolayers, were generally low and increased with time in culture (**Figure 4.6B**). Continuous culture in 2%  $O_2$  for 6, 12, and 18 days did not increase apoptosis, indicating that the lower cell numbers in hypoxic cultures were attributable to decreased proliferation, not increased cell death.

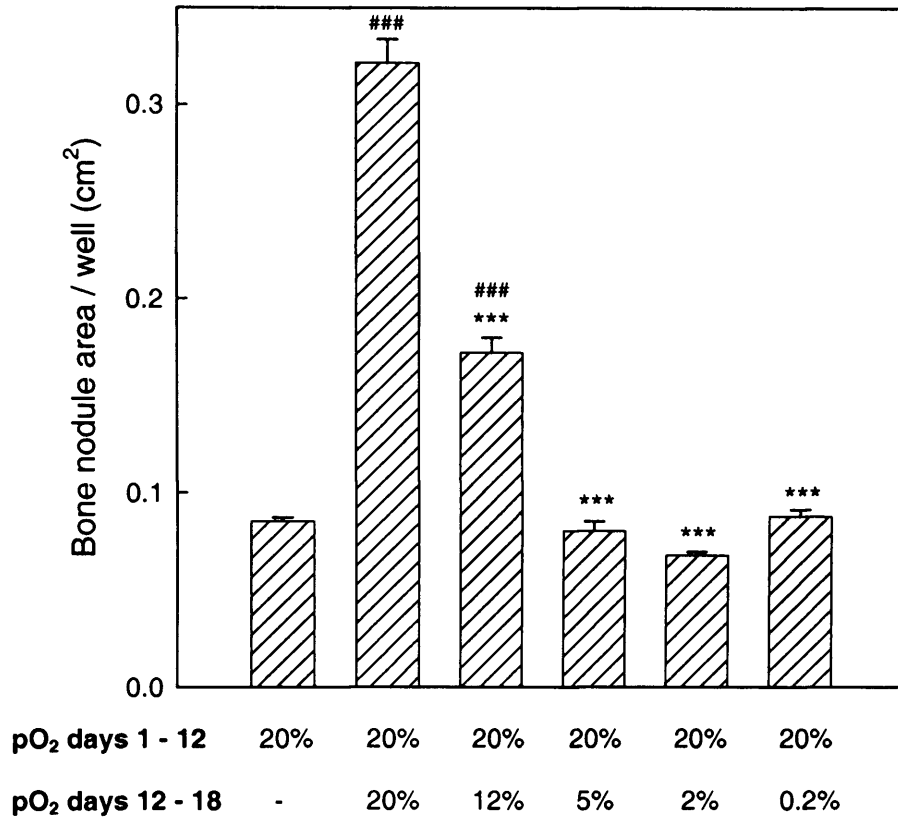
Alkaline phosphatase activity was greatly decreased in hypoxic osteoblasts during the mineralising phase of culture (**Figure 4.7**). Osteoblasts grown in 20%  $O_2$  showed a robust increase in alkaline phosphatase activity from day 6 to day 12 in culture, but in hypoxic cultures this increase did not occur.

### ***Effect of hypoxia on collagen ultrastructure and biochemistry***

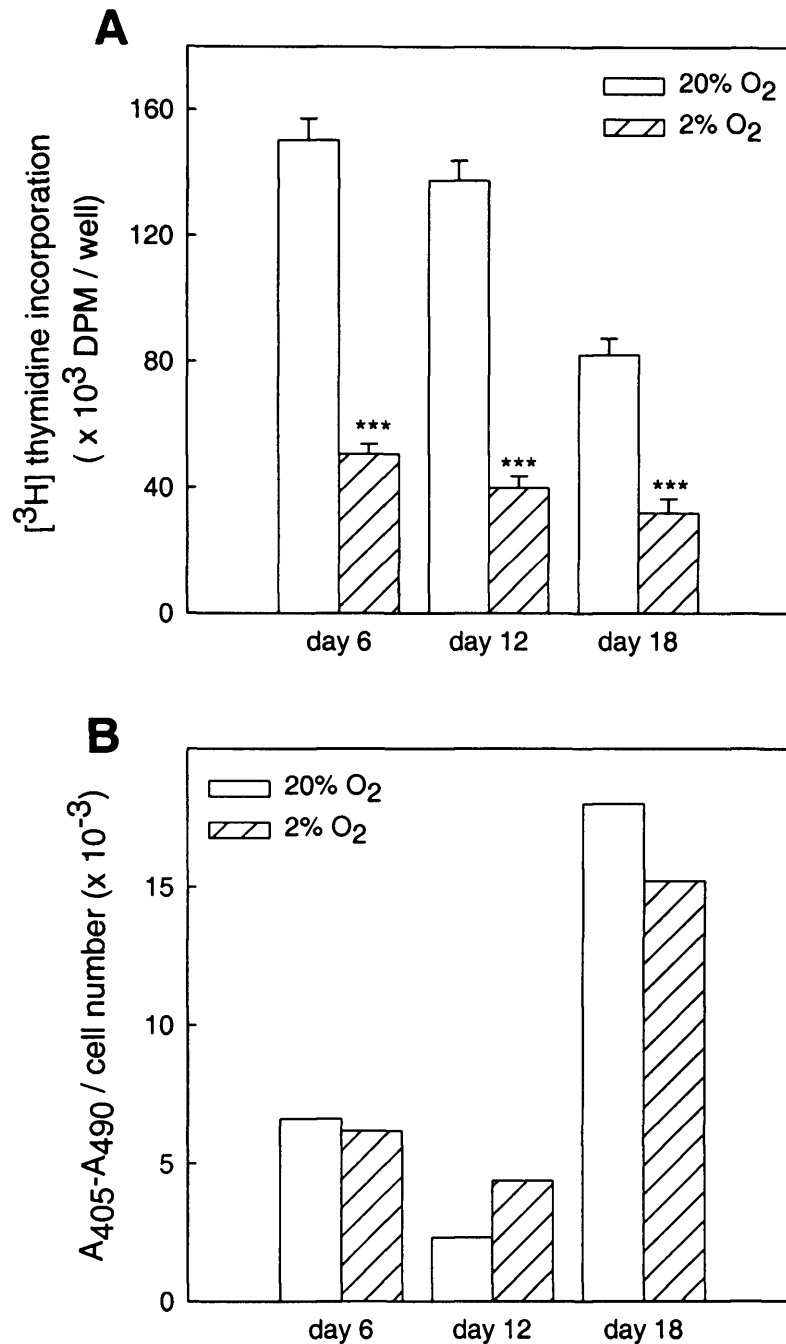
Because the enzymes involved in collagen formation utilise molecular oxygen for their activity, we examined the effect of hypoxia on the ultrastructure of collagen deposited in bone nodules by osteoblasts. Transmission electron microscopy revealed the presence of abundant, dense, regular bundles of collagen fibrils in normoxic (20%  $O_2$ ) cultures after 35 days. However, in cultures maintained for 35 days in 2%  $O_2$ , collagen fibrils were deposited at much lower density and in a less-organised manner (**Figure 4.8**).

We also found that the small amount of collagen deposited in hypoxic cultures was much more susceptible to pepsin degradation than collagen formed in 20%  $O_2$ : approximately 95% of collagen formed in 20%  $O_2$  was insensitive to pepsin,





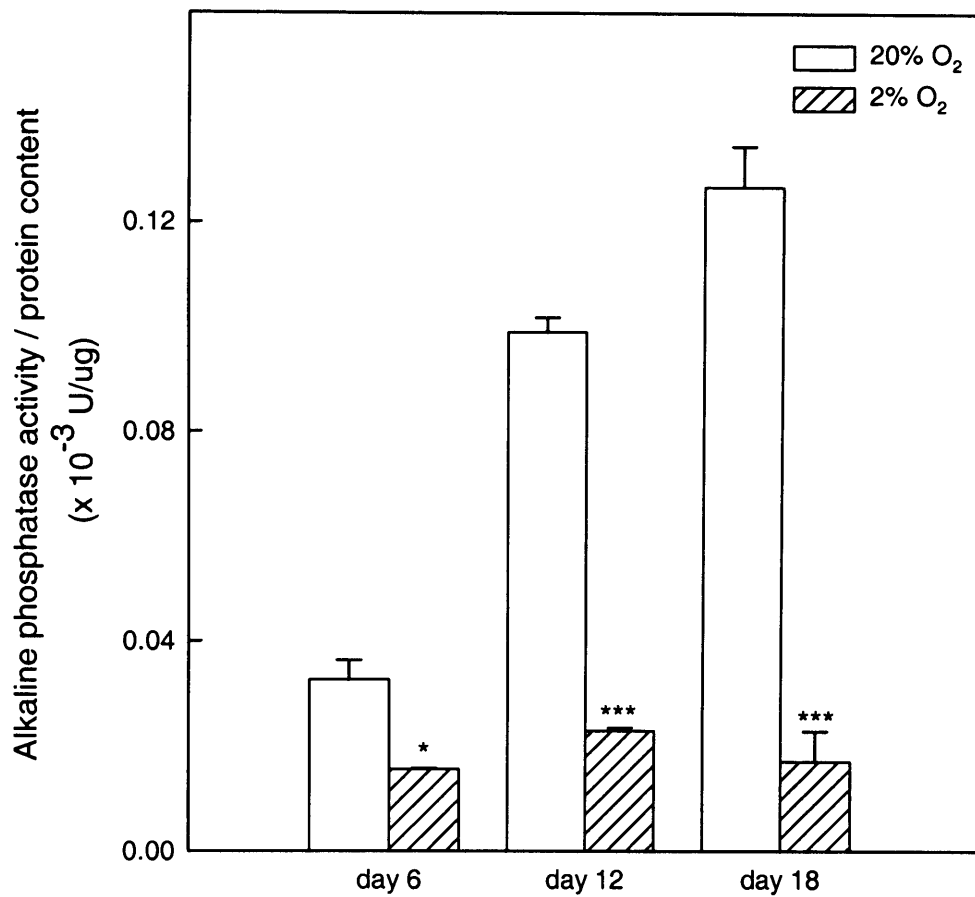
**Figure 4.5: Decreasing pO<sub>2</sub> inhibits nodule formation by mature osteoblasts.** Osteoblasts were formed in 20% O<sub>2</sub> for 12 days then transferred to lower pO<sub>2</sub> for the final 6 days of culture. Reducing the O<sub>2</sub> concentration led to significant inhibition of nodule formation compared to cells left at 20% O<sub>2</sub>. Below 12% O<sub>2</sub> nodule formation failed to increase over control cultures stopped at day 12 (20% / -), indicating complete inhibition of matrix formation. \*\*\* p < 0.001 vs. 20% O<sub>2</sub> / 20% O<sub>2</sub>; ### p < 0.001 vs. 20% O<sub>2</sub> / (-). Values are means ± SEM; n = 6.



**Figure 4.6: Hypoxia inhibits osteoblast proliferation but does not increase apoptosis.**

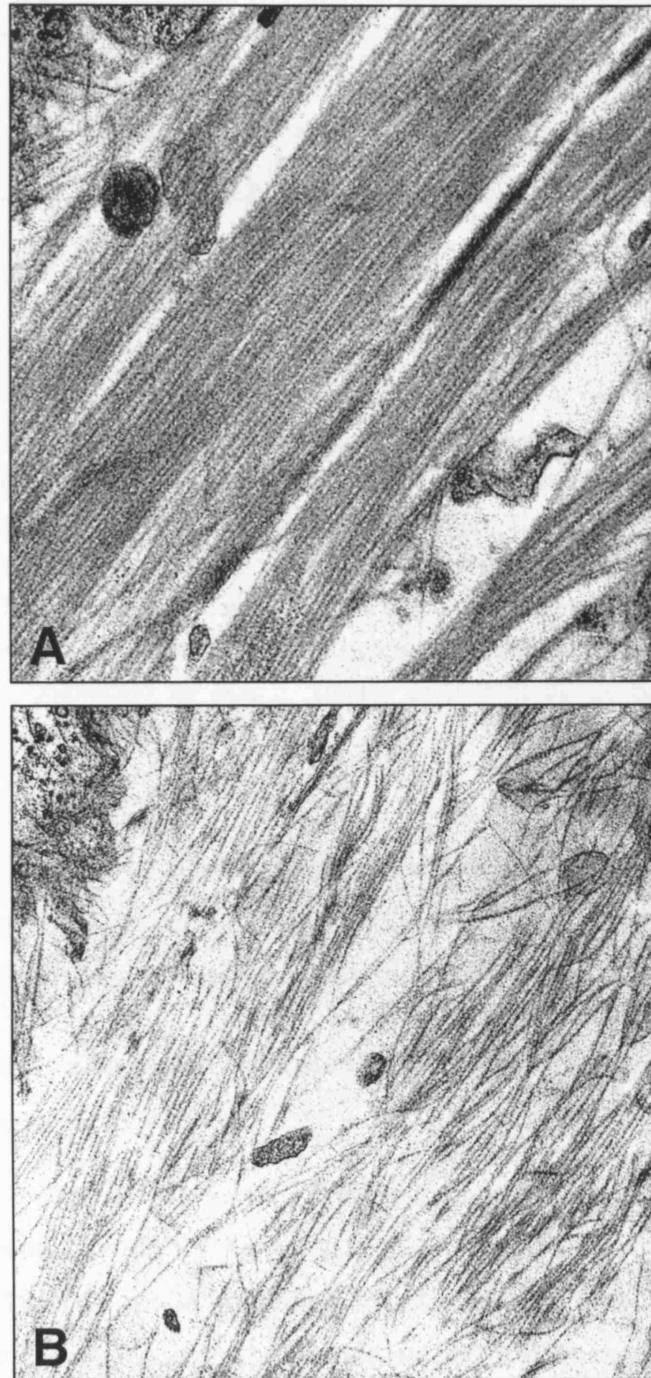
**A:** Hypoxia inhibits the proliferation of primary rat calvarial osteoblasts at day 6, 12 and 18 as measured by tritiated thymidine incorporation. \*  $p < 0.5$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  vs. 20% O<sub>2</sub>. Values are means  $\pm$  SEM;  $n = 6$ .

**B:** Osteoblast apoptosis increases with time in culture but is not induced by continuous exposure to low pO<sub>2</sub> (2%). Osteoblasts were grown in 20% or 2% O<sub>2</sub> for 6, 12 or 18 days before apoptosis was determined colorimetrically in pooled cell lysates using enzyme-linked immunosorbent assay (ELISA) that detects cytoplasmic histone DNA fragments; results are normalized to cell number. Values are means of duplicate determinations.



**Figure 4.7: Hypoxia inhibits alkaline phosphatase activity in rat calvarial osteoblasts.** Alkaline phosphatase activity, normalised to cell protein content, failed to increase from day 6 levels in osteoblasts grown continuously in 2%  $\text{O}_2$ . A robust increase was observed over the same time period for osteoblasts cultured in 20%  $\text{O}_2$ . \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  vs. 20%  $\text{O}_2$ . Values are means  $\pm$  SEM;  $n = 6$ .

**G**



**Figure 4.8: Hypoxia-induced changes in the ultrastructure of collagen fibrils.**

Transmission electron microscopy was performed on demineralised bone nodules formed by rat calvarial osteoblasts grown in 20% O<sub>2</sub> (A) and 2% O<sub>2</sub> (B). Collagen formed in 20% O<sub>2</sub> is deposited as dense, regular fibrils, whereas the collagen formed in low O<sub>2</sub> is characterised by a lower fibril density and a disorganised appearance. (Original magnification = 3000x).

Oxygen (%)	Collagen (nmol / ml)	Pepsin-insoluble collagen (%)	Lysine hydroxylation (%)	Proline hydroxylation (%)
20%	<b>4.96</b>	<b>94.9</b>	<b>31.6</b>	<b>43.9</b>
2%	<b>0.22</b>	<b>54.6</b>	<b>22.3</b>	<b>42.2</b>

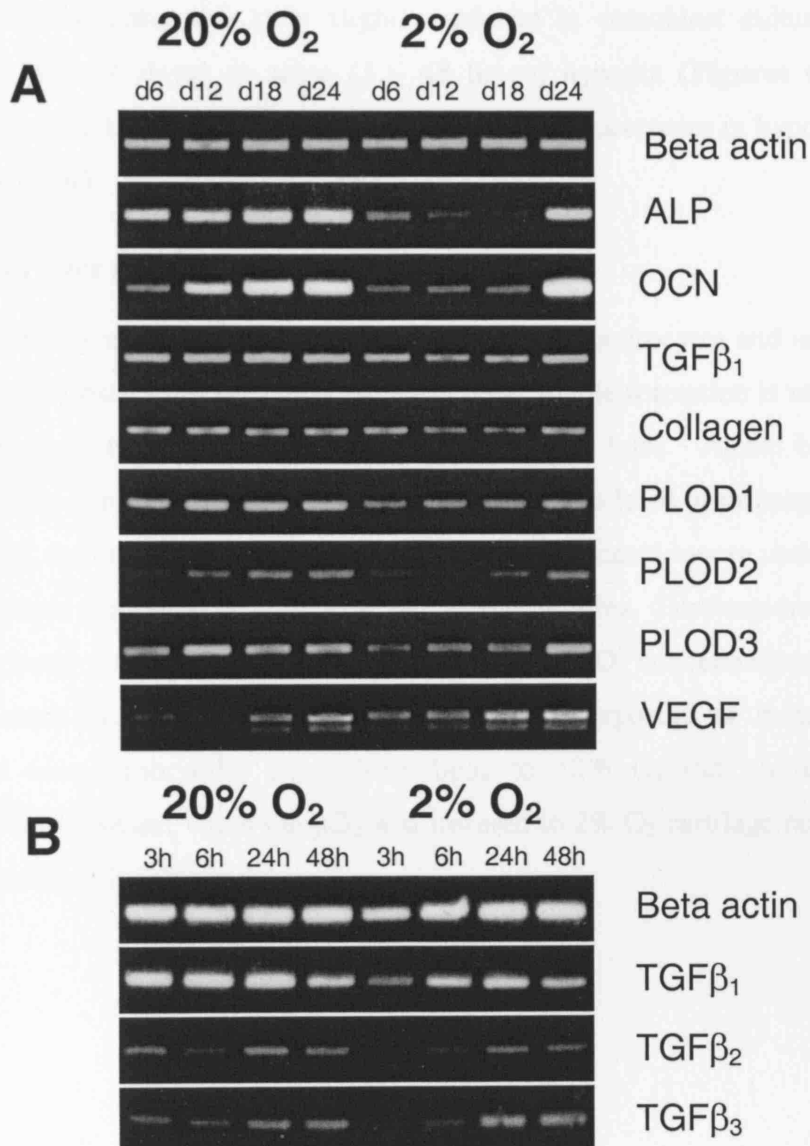
**Table 4.2: Hypoxia decreases collagen formation and lysine hydroxylation percentage.**

Collagen formed in osteoblast cultures at 20% and 2% O<sub>2</sub> was analysed to determine the percentage of hydroxyproline and hydroxylysine residues. Collagen formed by osteoblasts in hypoxia was less resistant to pepsin digestion than that formed in 20% O<sub>2</sub>, and hypoxic osteoblasts formed 25-fold less collagen. The percentage of hydroxylysine residues versus unmodified lysine residues was 1/3 less in hypoxic collagen, however proline hydroxylation percentage was unaffected by pO<sub>2</sub>. (Assay performed by Dr Simon Robins, Rowett Research Institute, Aberdeen).

compared to only 55% of 2% O<sub>2</sub> collagen (**Table 4.2**). Moreover, hydroxylation of lysine residues were decreased by about 30% in hypoxic collagen, although the proportion of hydroxyproline residues was unaltered.

#### ***Hypoxia delays expression of osteoblast markers and genes involved in collagen formation***

Observed delays in osteoblast differentiation in response to hypoxia (**Figure 4.9A**). Expression of mRNA for osteocalcin, a marker of mature osteoblasts, was strong by day 12 of culture in osteoblasts maintained in 20% O<sub>2</sub>, increasing further as cultures matured; however, in hypoxic cultures osteocalcin expression remained low until day 24 of culture. A similar effect of hypoxia was observed on the expression pattern of mRNA for alkaline phosphatase. The 3 procollagen lysine, 2-oxoglutarate, 5-dioxygenases (PLOD1-3) responsible for the formation of hydroxylysine residues were differentially expressed during osteoblast maturation. Hypoxia (2% O<sub>2</sub>) caused decreases in expression of all of the PLOD genes (**Figure 4.9A**), in line with the delay observed with ALP and OCN expression. In contrast, expression of mRNAs



**Figure 4.9: Hypoxia affects gene expression patterns in osteoblasts.**

**A:** RNA was extracted at days 6, 12, 18, and 24 and gene expression during osteoblast differentiation was investigated using RT-PCR. Long-term hypoxia (2% O<sub>2</sub>) leads to delayed expression of the mature osteoblastic differentiation markers alkaline phosphatase (ALP) and osteocalcin (OCN). Expression of the gene for type I collagen was relatively unaffected by hypoxia, as was transforming growth factor  $\beta$  (TGF $\beta$ ). Expression of the collagen modifying enzymes procollagen lysine, 2-oxoglutarate, 5-dioxygenase (PLOD) 1-3 were decreased by long-term culture in hypoxia. The angiogenic cytokine vascular endothelial growth factor (VEGF) is upregulated by hypoxia.

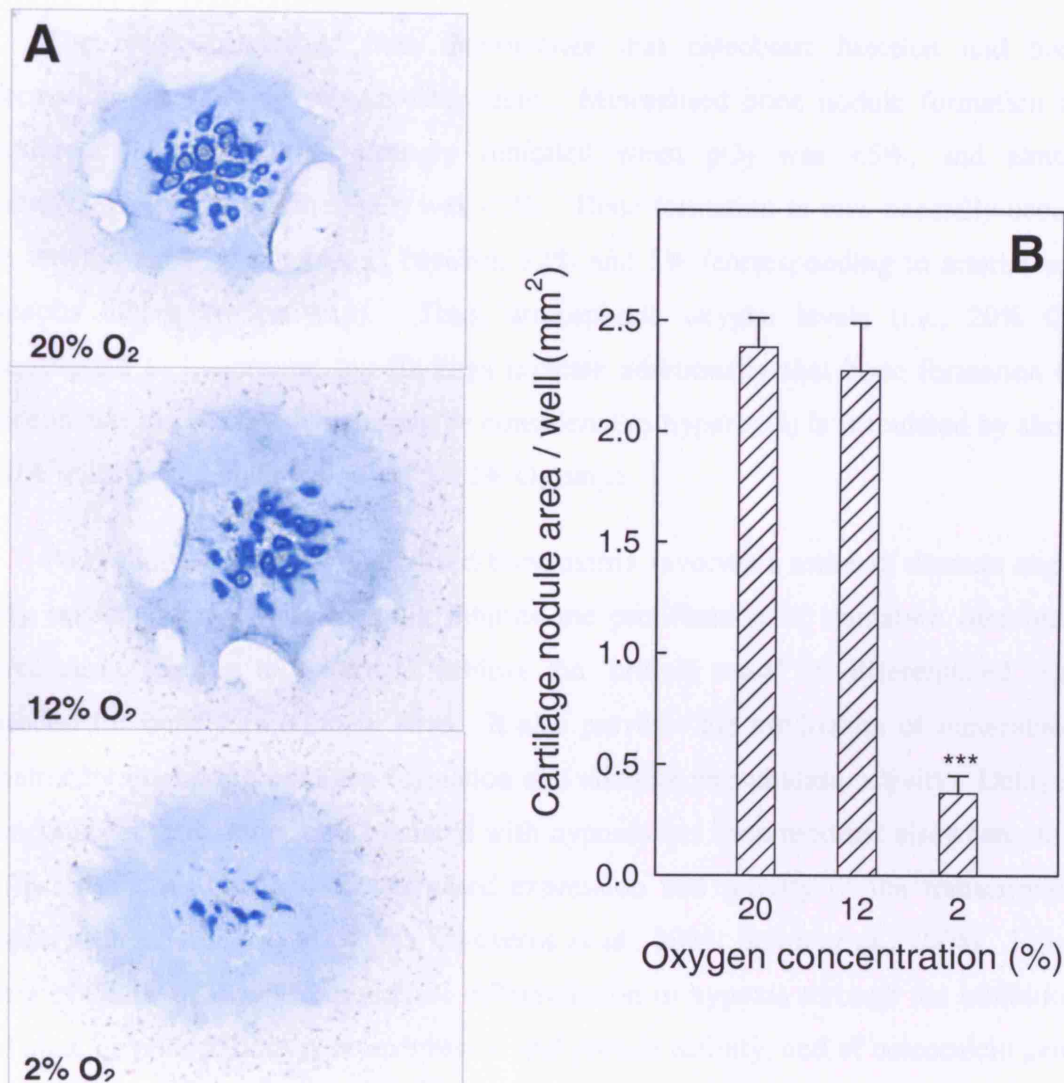
**B:** RNA was extracted following 3, 6, 24 and 48 hours exposure to controlled oxygen conditions, and gene expression was investigated using RT-PCR. Short-term exposure to 2% O<sub>2</sub> decreased the expression of TGF $\beta$  isoforms.

for TGF $\beta$  isoforms was only slightly reduced in osteoblast cultures exposed to chronic (6 – 24 days) or acute (3 – 48 hours) hypoxia (**Figures 4.9A & 4.9B**). Similarly COL1A1 mRNA levels showed only small decreases in hypoxic osteoblasts (**Figure 4.9A**).

### ***Hypoxia does not alter cell fate***

Mesenchymal cells can also differentiate into chondrocytes and adipocytes. The inhibition of osteoblast differentiation and bone nodule formation is not accompanied by increased differentiation to these alternative cell fates. Alcian blue staining of monolayers exposed to 2% O<sub>2</sub> reveal no change in the level of proteoglycans present. Similarly, staining of monolayers with Oil red O indicated low to undetectable levels of adipocyte formation in both 20% and 2% O<sub>2</sub> cultures. Furthermore, production of cartilaginous matrix was actively inhibited at lower O<sub>2</sub> concentrations when studied using chick micromass cultures (**Figure 4.10**). Exposure of mesenchymal cells derived from embryonic chick limb buds to 12% O<sub>2</sub> did not affect cartilage formation; however, when the pO<sub>2</sub> was lowered to 2% O<sub>2</sub> cartilage nodule formation was significantly inhibited.





**Figure 4.10: Hypoxia inhibits cartilaginous nodule formation in micromass cultures.** (This assay was performed in collaboration with Alex Lee)

**A:** Representative scanned images of alcian blue stained nodules formed in 20%, 12%, and 2% O<sub>2</sub>.

**B:** Area of cartilage nodules was quantitated in micromass cultures. Lowering the pO<sub>2</sub> to 2% caused significant inhibition of cartilage matrix production. \*\*\* p < 0.001. Values are mean ± SEM, n = 6.

## Discussion

The results presented here demonstrate that osteoblast function and bone formation are strongly oxygen-dependent. Mineralised bone nodule formation by cultured osteoblasts was strongly inhibited when  $pO_2$  was  $<5\%$ , and almost completely prevented when  $pO_2$  was  $<1\%$ . Bone formation *in vivo* normally occurs in environments where  $pO_2$  is between 12% and 5% (corresponding to arterial and venous blood, respectively). Thus, atmospheric oxygen levels (*i.e.*, 20%  $O_2$ ) correspond to hyperoxia; my findings indicate additionally that bone formation by osteoblasts in 20%  $O_2$  (which may be considered as hyperoxia) is stimulated by about 50% relative to the physiological 5-12%  $O_2$  range.

Formation of mature, mineralised bone matrix involves a series of discrete steps. My results indicate that hypoxia inhibits the proliferation of immature osteoblast precursors, leading to failure to achieve the 'critical mass' of differentiated cells needed for bone formation *in vitro*. It also prevents the production of mineralised matrix by disrupting collagen formation and alkaline phosphatase activity. Delayed osteoblastic differentiation associated with hypoxia has been reported elsewhere; this effect has been ascribed to decreased expression and activity of the transcription factor, Runx2 (Park *et al.*, 2002; Ontiveros *et al.*, 2004; Salim *et al.*, 2004). I also saw evidence of delayed osteoblast differentiation in hypoxia through the inhibition of alkaline phosphatase gene expression and protein activity, and of osteocalcin gene expression. A previous report described up-regulation of collagen and TGF $\beta$  gene expression (Warren *et al.*, 2001) in short-term cultures of rat calvarial osteoblast cultures exposed to  $\sim 5\%$   $O_2$ ; in the present study I observed no obvious changes in gene expression of either collagen or TGF $\beta$  in short-term osteoblast cultures exposed to 2%  $O_2$ , and modest decreases in longer-term cultures.

Cartilage is an avascular tissue and maintenance of the chondrocyte phenotype is encouraged by decreased  $pO_2$  (Domm *et al.*, 2002). To investigate whether hypoxia might promote the formation of cartilage, production of cartilaginous nodules in micromass cultures of mesenchymal cells from embryonic chick limb buds was investigated. In 2%  $O_2$ , cartilage nodule formation was reduced by 5-fold; this

inhibition was thus of similar magnitude to that observed for bone nodule formation in hypoxia. Cartilage formation in rat osteoblast cultures was investigated by alcian blue staining; however, very few such nodules were observed. There was no observable increase in alcian blue staining in hypoxic osteoblast cultures, which was consistent with results obtained in chick micromass cultures. In a recent study the formation of bone and cartilage by adipose derived mesenchymal cells in low pO<sub>2</sub> (2%) was investigated; in agreement with my findings they noted reduced osteogenesis and chondrogenesis in hypoxic conditions (Malladi *et al.*, 2005). Similarly, I was unable to find any evidence for increased adipogenesis in osteoblast cultures exposed to low oxygen using oil red O staining. This result is consistent with the reports that hypoxia inhibits proliferation, differentiation and adipogenesis by adipocytes (Kim *et al.*, 2005; Sahai *et al.*, 1994).

The results presented here suggest that hypoxia may exert important inhibitory effects on osteoblast collagen production at the post-translational level. Hydroxyproline residues within collagen provide thermal stability, enabling correct intracellular triple helix formation, whereas hydroxylysine residues provide covalent linkage sites between adjacent collagen molecules within deposited fibrils. The enzymes responsible for proline and lysine hydroxylation are oxygen-dependent (Kagan and Li, 2003; Tandara and Mustoe, 2004). The collagen-specific prolyl-4-hydroxylase (P4OH) requires 20mmHg O<sub>2</sub> (~3%) to display 50% of its maximal activity and 150mmHg O<sub>2</sub> (~20%) for 90% of maximal activity (Tandara and Mustoe, 2004). Considering the central role of prolyl hydroxylases in oxygen sensing (Epstein *et al.*, 2001), it might be expected that the proportion of hydroxylated proline residues would be decreased in collagen formed by hypoxic osteoblasts. However, collagen molecules with insufficient proline hydroxylation are unstable at body temperature, and would not proceed to the triple helical conformation and deposition (Fessler and Fessler, 1974). Underhydroxylated procollagen is retained intracellularly with some degradation, and upon resumption of permissive conditions for hydroxylation this procollagen is then processed normally (Ramaley and Rosenbloom, 1971). These findings suggest that if proline hydroxylation is compromised in hypoxia, it would be difficult to detect such changes in any collagen deposited; rather, the phenomenon would present as a generalised decrease in total

collagen. Such a mechanism would also be consistent with the reversibility of the hypoxic inhibition of collagen deposition.

The decreased percentage of hydroxylysine residues in hypoxia may reflect the requirement of the PLOD enzymes for molecular oxygen (Kikuchi *et al.*, 1983), together with the observed reduction in PLOD expression in the hypoxic cultures. These findings contrast with reported increases in PLOD-2 expression during hypoxic culture of dermal fibroblasts (Brinckmann *et al.*, 1999) and increased PLOD-1 and -2 gene expression in muscle cells in response to hypoxia *in vitro* (Hofbauer *et al.*, 2003). The changes in pepsin sensitivity of the collagen formed by hypoxic osteoblasts could be accounted for by decreased activity of lysyl oxidase (LOX), and thus decreased covalent cross-linking (Kagan and Li, 2003). This notion is supported by the report that collagen isolated from rats treated with the specific LOX inhibitor,  $\beta$ -aminopropionitrile (BAPN) had increased sensitivity to pepsin digestion (Oxlund *et al.*, 1995). Decreased activity of LOX, leading to impaired intermolecular cross linking could also help to account for the disorganised collagen ultrastructure observed by transmission electron microscopy. Decreased collagen cross-linking has clear implications for bone strength and has been associated with osteoporosis (Oxlund *et al.*, 1995; Oxlund *et al.*, 1996).

Fibroblasts have been reported to increase their production of collagen when exposed to hypoxia *in vitro* (Falanga *et al.*, 2002). It is not easy to reconcile the reported fibroblast response to hypoxia with that observed for osteoblasts; however, it should be noted that no long-term investigations into collagen production or hydroxylation of residues in hypoxia have been published for fibroblasts. *In vivo*, however, wound-healing evidence suggests that collagen deposition is related directly to wound oxygen tension (Tandara and Mustoe, 2004).

A well-documented response of osteoblasts to hypoxia is upregulation of the potent angiogenic agent, vascular endothelial growth factor (VEGF) (Akeno *et al.*, 2001; Steinbrech *et al.*, 1999). These responses could be mediated via the stabilisation of HIF1 $\alpha$  and HIF2 $\alpha$  that occurs in hypoxic osteoblasts *in vitro* (Akeno *et al.*, 2002; Kim *et al.*, 2002). VEGF is required for fracture healing (Street *et al.*, 2002), and is essential for normal bone development (Zelzer *et al.*, 2002).

Osteoblasts express VEGF receptors and VEGF increases mineralised nodule formation in osteoblast cultures (Deckers *et al.*, 2000; Street *et al.*, 2002). However, despite the increased VEGF expression observed in hypoxic osteoblasts, bone nodule formation was inhibited strongly by hypoxia in my cultures, suggesting that in the absence of sufficient oxygen, osteoblasts cannot mount an anabolic response to VEGF. *In vivo*, however, hypoxia would induce expression of VEGF (and other factors), leading to angiogenesis and, once sufficient oxygen was available, increased osteoblastic bone formation.

Bone nodule formation by osteoblasts was susceptible to inhibition by hypoxia at both early and late stages of culture, but recovered well when cells were transferred to atmospheric oxygen levels following early exposure to hypoxia. Osteoblast apoptosis was low, but increased slightly with time in culture, in line with another recent study (Brandao-Burch *et al.*, 2005); surprisingly, however, long-term hypoxia did not increase apoptosis. These observations are consistent with the notion that hypoxia induces a state of quiescence or ‘suspended animation’ in osteoblasts. It seems reasonable to suppose that such a response to hypoxia, which could be mediated via the involvement of HIF proteins (Maxwell, 2002), would serve to ensure osteoblast survival *in vivo* until (re)vascularisation occurred.

Hypoxia *in vivo* also results in local decreases in tissue pH due to increased anaerobic metabolism and reduced perfusion. We recently showed that mineralisation of organic matrix deposited by osteoblasts is extremely sensitive to inhibition by small pH reductions (although osteoblast proliferation and collagen production were unimpaired) (Brandao-Burch *et al.*, 2005). Therefore, culture medium acidification was carefully controlled in order to eliminate pH as a variable. The inhibitory response of osteoblasts to hypoxia is reciprocal with the powerful stimulatory action of hypoxia on osteoclast formation (and thus, bone resorption). It is noteworthy that even in severe, chronic hypoxia (0.2% O<sub>2</sub>), mouse or human osteoclast formation is increased 2-3 fold compared with 20% O<sub>2</sub> (Arnett, 2003; Utting *et al.*, 2004); whereas osteoblast function is almost completely blocked in such conditions. The results presented here show that bone formation by osteoblasts *in vitro* is critically dependent on oxygen concentration.

# CHAPTER 5

## *General Discussion*

The role of the vasculature in controlling the function of bone cells has been relatively neglected. It has been known since Havers made his observations on the blood supply within bones that bone is not a 'dead' tissue, that it is perfused by blood vessels and that the cells within bone require this supply to survive. The requirement for oxygen is common to all cells in the body, but the response to withdrawal of oxygen varies enormously between different types of cells.

I have shown that hypoxia induces the formation of osteoclasts from bone marrow precursors in rodents and monocytic precursors in humans. The osteoclasts formed in hypoxia are larger and, correspondingly, are capable of resorbing more bone (or dentine). Although hypoxia does not directly stimulate the resorptive activity of mature osteoclasts, it causes acidosis *in vivo* and *in vitro*, which will then activate osteoclasts directly. The hypoxia effect requires the presence of a supply of M-CSF and RANKL, and may be mediated, at least in part, by increased production of the osteoclastogenic factors VEGF and IL-6. I have shown that osteoclasts express the hypoxia transcription factors HIF1 $\alpha$  and 2 $\alpha$  and stabilise these in response to hypoxia and cobalt ions which mimic hypoxia. Further investigation of the intracellular signalling pathways controlling these effects is currently under way.

My work on the effects of hypoxia on osteoblasts revealed multiple levels of regulation of osteoblast function by oxygen tension. Hypoxia inhibited the formation of mineralised bone nodules in cultures of primary rat calvarial osteoblasts in a 'dose-dependent' manner. This inhibition was caused through a combination of decreased cell proliferation and decreased matrix production. Hypoxia also inhibited or delayed the expression of alkaline phosphatase and osteocalcin, both markers of the mature osteoblast phenotype. Hypoxia did not cause increases in the apoptosis

rate in osteoblast cultures at any stage but appeared to induce, in effect, a state of reversible 'suspended animation'. Osteoblast differentiation was delayed in hypoxic osteoblast cultures, and the amount of collagenous matrix formed in hypoxic cultures was greatly reduced compared to that formed by osteoblasts cultured in 20% O<sub>2</sub>. Hydroxylation of lysine residues in the deposited collagen fibrils was diminished in 2% O<sub>2</sub>, and the collagen fibrils formed by osteoblasts in hypoxic conditions were less dense and more disorganised. The effects of hypoxia on differentiation were separate from those on nodule formation, as exposure of mature osteoblasts to decreased pO<sub>2</sub> caused strong inhibition of nodule formation. The effects of hypoxia on osteoblasts were comparable with its inhibitory effects on chondrocyte matrix deposition in chick limb-bud micromass cultures.

The osteoclast and the osteoblast, the two main cell types in bone, are derived from two separate lineages. As my results have shown, these cells respond in completely different ways to a hypoxic insult, with the net result indicating that hypoxia would have a severely detrimental impact on bone. The responses elicited by hypoxia *in vitro* are consistent with *in vivo* observations of bone loss at sites of blood vessel disruption, and have significant implications for the future path of investigations in bone biology. The first issue that this work raises is methodological. Contrary to tradition, atmospheric oxygen should not be assumed normal for tissue culture experiments. *In vivo* tissue oxygen tensions range from approx 5 to 12%, and in pathological situations are considerably lower than this (Lewis *et al.*, 1999; Vaupel *et al.*, 1989). As my work has shown, lowering pO<sub>2</sub> is capable of dramatically altering cell differentiation and function. The simple, low-cost system that I used to create and maintain low oxygen environments could be used easily for cell culture experiments in other fields.

Very few studies have measured oxygen tensions within the bone microenvironment. This is due to the difficulty of inserting probes into mineralised tissue. New techniques such as magnetic resonance imaging are now becoming available; however, these have limitations in that they detect changes in haemoglobin / deoxyhaemoglobin levels rather than measuring pO<sub>2</sub> directly (Gallez and Mader, 2000; Swartz, 2002). The *in vitro* results I have presented may give an indirect indication of bone pO<sub>2</sub> *in vivo*. It seems reasonable to assume that within the bone a

balance needs to be achieved between bone formation by osteoblasts, and bone resorption by osteoclasts; the cells must be sensitive to changes in local or systemic oxygenation to maintain bone mass. If this assumption is correct then my results indicate that the oxygen tension that provides a balance between formation and resorption is between 5% and 12% O<sub>2</sub>; this correlates well with the few bone marrow oxygen measurements that have been published (Harrison *et al.*, 2002; Ishikawa and Ito, 1988). Bone pO<sub>2</sub> will vary within the bone and also depending on the location of the bone in the body. The pO<sub>2</sub> of the calvaria in neonatal rats could be higher, due to its proximity to the surface of the skin.

Although it has long been known that bone requires a vascular supply, the role of this supply in regulating the fundamental parameters, pO<sub>2</sub> and pH was not appreciated. My work gives an indication that supply of oxygen via the vasculature is vital to the formation and activity of the cells within bone. These results may help to clarify the role of VEGF, the major angiogenic stimulus induced by hypoxia (Akeno *et al.*, 2001; Akeno *et al.*, 2002; Bouletreau *et al.*, 2002; Kim *et al.*, 2002; Komatsu and Hadjiargyrou, 2004; Steinbrech *et al.*, 1999; Steinbrech *et al.*, 2000), in endochondral ossification (Maes *et al.*, 2002) and healing of fractured bone (Street *et al.*, 2002). Loss of a single VEGF isoform leads to decreased vascularisation and oxygen delivery, and subsequently, a lack of bone formation; loss of all VEGF isoforms results in embryonic lethality (Zelzer *et al.*, 2002). VEGF has roles independent of oxygen delivery, as it has been shown to directly affect osteoclasts and osteoblasts at atmospheric pO<sub>2</sub> (Aldridge *et al.*, 2005; Street *et al.*, 2002); however, its stimulatory activity may be hindered in the absence of sufficient oxygen.

My findings cast light onto some of the mechanisms by which bone loss could occur during a number of pathological situations. Inflammation and infection lead to localised hypoxia, hypoxia also induces the release of a large number of pro-inflammatory mediators; this leads to a vicious cycle of hypoxia and inflammation (Murdoch *et al.*, 2005). In both rheumatoid and osteoarthritis pO<sub>2</sub> in the synovial fluid is decreased (Giatromanolaki *et al.*, 2003; Taylor and Sivakumar, 2005). This is accompanied by stabilisation of HIF1 $\alpha$ , and the release of angiogenic factors including VEGF (Berse *et al.*, 1999; Clavel *et al.*, 2003; Giatromanolaki *et al.*, 2003).



My results indicate that the decreased  $pO_2$  would directly induce osteoclast formation, and the accompanying inflammatory acidosis would enhance the activity of osteoclasts present at the site.

Bone fracture sites are hypoxic; in rabbits, measurement of oxygen tension in the haematoma 4 days post-fracture gave average values of 0.8%  $O_2$ , and by 14 days post fracture this had increased to 3.8%  $O_2$  (Brighton and Krebs, 1978). This environment would be profoundly inhibitory to bone formation, whilst osteoclast formation and activity would be stimulated by the combination of hypoxia and acidosis. The initial enhanced osteoclast activity is consistent with the requirement to remove broken bone fragments and clean the site prior to new bone formation. The angiogenesis stimulated by hypoxia, through the release of cytokines such as VEGF, is essential for fracture healing, as it allows the delivery of oxygen and removal of waste products from the fracture site. Delivery of oxygen to the site of fracture using hyperbaric oxygen therapy (HBOT) has been shown to improve fracture healing in clinical trials (Bouachour *et al.*, 1996); and bone formation in animal models of non-union fracture (Kerwin *et al.*, 2000). My results suggest that this treatment would have direct effects on osteoblast mediated bone formation.

Like many tumours, invasive breast cancers are hypoxic and exhibit high levels of angiogenesis associated with HIF1 $\alpha$  stabilisation (Vaupel *et al.*, 1988; Bos *et al.*, 2005). Although these tumours are highly vascularised, hypoxia is common due to the high metabolic rate of the cancer cells and the disorganised and 'leaky' nature of the tumour vasculature. Metastasis of breast cancer to bone is common, and these osteolytic tumours are highly vascularised and cause extensive bone loss (Shimamura *et al.*, 2005). The bone loss accompanying tumour metastasis could be at least partly due to enhanced osteoclastogenesis and reduced osteoblast formation and activity caused by the hypoxic environment created by the tumour within the marrow.

Patients with chronic obstructive pulmonary disease (COPD) experience systemic hypoxia (Franciosi *et al.*, 2004) and have a higher fracture risk (de Vries *et al.*, 2005); the increased fracture risk could be partially accounted for by systemic hypoxia. The situation may be exacerbated in COPD patients by glucocorticoid treatment, as this has been associated with bone loss (Dempster *et al.*, 1989). Glucocorticoids have

direct actions on bone cells (reviewed in Reid, 1997); however, these drugs also increase circulating endothelin-1 levels, and this is a potent vasoconstrictor (Borcsok *et al.*, 1998).

Disuse induced bone loss has been linked to osteocyte hypoxia. Osteocytes are encased in bone, often remote from blood vessels, and must thus derive their nutrients from fluid flow during mechanical stimulation (Knothe Tate and Knothe, 2000; Wang *et al.*, 2000). Dodd and colleagues (1999) have reported that HIF1 $\alpha$  is stabilised in osteocytes in bone subject to disuse. The release of angiogenic factors such as VEGF by osteocytes during these periods of hypoxia could potentially regulate osteoclast activity at the surface of the bone.

The bone marrow is the main adult site of immune cell production. It has recently been proposed that certain cells within the bone marrow constitute a stem cell 'niche' by providing essential molecular adhesions, such as N-cadherin, that allow haematopoietic stem cells to survive in an undifferentiated state (Zhang *et al.*, 2003). The movement from multipotent stem cell to committed progenitor is associated with the loss of these cell adhesions. The niche supporting cells are thought to be the bone lining cells, thought to be dormant osteoblasts (Calvi *et al.*, 2003). It is known that haematopoietic cells proliferate and survive better in low oxygen culture conditions, and that this also helps to maintain their multipotency (Ivanovic *et al.*, 2000; Ivanovic *et al.*, 2002; Ivanovic *et al.*, 2004). Bone marrow modelling studies indicate that the conditions at the periphery of the bone marrow are hypoxic (Chow *et al.*, 2001); this would allow for the maintenance of stem cell multipotency. My results for the effect of hypoxia on osteoblasts are consistent with a 'dormant' phenotype; thus, it is possible that the bone lining cells that support the stem cell niche are simply hypoxic osteoblasts. Interestingly, mice deficient in HIF2 $\alpha$  have altered haematopoiesis associated with a defect in the supporting bone marrow stromal cells (Scortegagna *et al.*, 2003). Investigation of the phenotype of osteoblasts in the HIF2 $\alpha$  knockout animals, and their response to hypoxic insult, and the expression of markers of the 'niche' maintaining bone lining cell, such as N-cadherin, in hypoxic osteoblast cultures would help to test this hypothesis.

HIF1 $\alpha$  knockout mice do not survive past embryonic day 11 (Iyer *et al.*, 1998; Kotch *et al.*, 1999; Ryan *et al.*, 1998); hence, the effect of the loss of HIF1 $\alpha$  activity on bone has not been studied. Macrophage and neutrophil formation and activation require the presence of HIF1 $\alpha$ ; regardless of oxygen tension, HIF1 $\alpha$  is vital for energy production (Cramer *et al.*, 2003). It is possible that this could also be the case for osteoclasts. Bone resorption is an energy intensive process, and osteoclasts have a large number of mitochondria (Williams *et al.*, 1997). Despite this, osteoclasts formed in low oxygen resorb bone avidly. The stabilisation of HIF1 $\alpha$  mediates the expression of enzymes required for anaerobic glycolysis (Hu *et al.*, 2003), therefore, loss of HIF1 $\alpha$  could potentially abrogate bone resorption in hypoxia.

Tooth movement requires the activity of osteoclasts to degrade the bone of the jaw, and is achieved by applying force to the tooth in the direction of movement. The mechanism responsible for this force-induced movement is not clear (Roberts-Harry and Sandy, 2004); however, the force required to move a tooth is sufficient to impede the blood supply to the bone matrix under pressure and make this area hypoxic, as determined by decreased respiration of pulp tissue under pressure during tooth movement (Hamersky *et al.*, 1980). This hypoxic environment would favour osteoclast formation, leading to bone removal and tooth movement.

Evidence for the central regulation of bone mass via the peripheral nervous system is beginning to accumulate. Several factors, such as leptin (Ducy *et al.*, 2000) and IL-1 (Bajayo *et al.*, 2005), have recently been implicated in controlling bone mass through the hypothalamus. Bone is highly innervated; nerves run along vessels adjacent to trabecular bone (Serre *et al.*, 1999). Systemic hypoxia, sensed by chemoreceptors in the carotid body and neuroepithelial cells in the lung, leads to adaptive responses in the cardiorespiratory system, such as hyperventilation. These effects are controlled through the respiratory centre in the brain and the peripheral nervous system (Lopez-Barneo *et al.*, 2004). The association between nerves and the blood vessels in the bone may allow for central regulation of the response to hypoxia in bone.

Many enzymatic reactions within cells require molecular oxygen as a substrate. The oxygen-regulated stability of the HIF $\alpha$  proteins has been much studied of late;

however, as already mentioned, collagen production involves the activity of at least 3 enzymes that rely on oxygen for their activity: these are prolyl-4-hydroxylase (Counts *et al.*, 1978), lysyl hydroxylase (Kikuchi *et al.*, 1983), and lysyl oxidase (Williamson *et al.*, 1986). Type 1 collagen is also produced by fibroblasts; a number of studies suggest that hypoxia induces fibroblastic collagen production (Agocha *et al.*, 1997; Falanga *et al.*, 2002; Norman *et al.*, 2000; Saed *et al.*, 2002). However, these studies examined mRNA expression only or collagen production after short-term (24-72 hour) incubation in hypoxia. The long-term effects of hypoxia on collagen synthesis in fibroblasts remain undetermined. Bearing in mind my findings for osteoblasts, a cell type closely-related to fibroblasts, and the oxygen dependent nature of the collagen modifying enzymes, it seem likely that fibroblast growth and collagen production would also be subject to chronic inhibition by hypoxia. This hypothesis would have important implications for wound healing, and might help to explain, for example, why diabetic patients experiencing peripheral artery occlusive disease develop chronic non-healing ulcers (Faglia *et al.*, 1998).

The enzymes nitric oxide synthase and cyclooxygenase, which produce the molecules NO and PGE<sub>2</sub> respectively, require oxygen for their activity (Kwon *et al.*, 1990; Lands *et al.*, 1978). The production of steroid hormones also utilises molecular oxygen (reviewed in Estabrook, 2005). The efficient production of ATP through the electron transport chain in mitochondria greatly depends upon oxygen supply, and ATP itself is utilised as a signalling molecule (Burnstock, 1987). It is reasonable to assume that long-term hypoxia will have detrimental effects on the production of these factors; the ramifications of this on the bone tissue are unknown.

The non-steroidal anti-inflammatory drugs (NSAIDs) exert potent inhibitory actions on COX activity and subsequent prostaglandin production. These drugs are commonly prescribed for alleviation of musculoskeletal pain associated with inflammation, but their use has been associated with delayed healing and increased risk of fracture non-union (reviewed in Wheeler and Batt, 2005). PGE<sub>2</sub> is a potent vasodilator and inhibition of its synthesis would decrease blood flow to the fracture site and inhibit the activity of osteoblasts. PGE<sub>2</sub> release in response to hypoxia would also be inhibited, preventing the increased resorption that accompanies it. Inflammation at sites of fracture would enhance the production of the angiogenic

cytokines IL-1, and TNF $\alpha$  (Lewis *et al.*, 1999). Inhibition of the inflammatory cascade/process would be thus detrimental to the process of fracture healing because the angiogenesis and remodelling of the fracture callus that is required for successful healing would be abrogated (Flick *et al.*, 2003).

### ***Future work***

The work presented in this thesis identifies hypoxia as a strong regulator of bone cell formation and activity *in vitro*. Further studies can now dissect the mechanism by which oxygen tension regulates bone cell activity. My current studies aim to confirm the presence or absence of HIF $\alpha$  stabilization in hypoxic osteoclasts and osteoblasts. This will be determined by immunofluorescence staining of monolayer cultures and Western blotting of cell lysates. The effects of hypoxia in the absence of a functioning HIF signalling system will be studied using RNA interference to knockdown gene expression of HIF1 $\alpha$  and HIF2 $\alpha$ . HIF1 $\alpha$  knockout animals die during embryonic development (Iyer *et al.*, 1998; Kotch *et al.*, 1999; Ryan *et al.*, 1998) however a myeloid cell specific knockdown of HIF1 $\alpha$  has been successfully produced indicating the feasibility of this approach (Cramer *et al.*, 2003). The production of the HIF2 $\alpha$  knockout mouse (Scortegagna *et al.*, 2003a) also offers opportunities to study the relative importance of this protein on total bone mass, and also in individual cell assays. It is possible that the observed effects of hypoxia are occurring independent of the HIF signalling pathway. The effect of hypoxia on calcium flux in osteoclasts in response to hypoxia, and the expression and activity of oxygen sensitive potassium channels in both osteoclasts and osteoblasts will be investigated using calcium-binding dyes.

Microarray analysis of osteoclast cultures, osteoblast cultures, and whole calvarial cultures to investigate gene expression changes induced by hypoxia would give insight into the signalling pathways activated in hypoxic cells. Real time PCR analysis of gene expression changes identified by reverse transcriptase PCR is currently under way. Defined protein arrays, for example targetting angiogenic and inflammatory mediators, are also commercially available, which could confirm that increased gene expression leads to increased protein production, and identify factors that are not transcriptionally mediated.

The stimulatory effects of hypoxia on osteoclast formation and activity raise interesting questions regarding their source of energy. They contain large numbers of mitochondria; what is the role of these mitochondria during oxygen deprivation? Collaborative studies are being initiated that will investigate the manner in which osteoclasts utilise energy resources in hypoxia and normoxia.

The requirement for molecular oxygen by the prolyl-4-hydroxylase and lysyl hydroxylase enzymes led to inhibition of collagen formation in hypoxic osteoblast cultures. Hypoxia has been implicated in fibrosis (Norman *et al.*, 2000); however, collagen production by fibroblasts during long-term hypoxia has not been clearly established, and relative levels of residue hydroxylation have not been studied. The effect of oxygen tension on fibroblastic collagen production, utilising the methods demonstrated for osteoblast collagen synthesis, could give insights into the process of scar formation, and non-healing ulcers in diabetics.

These *in vitro* cell culture based assays demonstrate the effects of hypoxia on individual cell types in simplified systems, but also provide potential insights into the effects of oxygen on complex bone organ systems *in vivo*. The circumstantial evidence for the effects of hypoxia on bone loss *in vivo* is strong; however, investigating these effects in a carefully controlled manner *in vivo* is difficult and expensive. Several methods could be used to induce hypoxia. Partial ligation of the femoral artery is a common mechanism of inducing ischaemia to the lower limbs; however, this is a very invasive technique and introduces complications due to the surgery and the inflammation that accompanies it. For example, the pain caused by the procedure will cause the animal to favour the contralateral limb. The hind limb suspension model could also be used; however, although blood flow is altered using this method (Colleran *et al.*, 2000), it is not clear how this model affects oxygen tension. Another possibility would be to chronically reduce the inspired pO<sub>2</sub> level of animals and monitor arterial blood oxygenation and bone turnover markers. If the model animal used was sufficiently large then bone marrow aspirates could also be tested for pO<sub>2</sub>. However, *in vivo*, low inspired pO<sub>2</sub> induces erythropoiesis as a compensatory measure (Hunter *et al.*, 1974), and how systemic alterations in pO<sub>2</sub> would affect bone pO<sub>2</sub> is unknown.

## **Conclusion**

The work presented in this thesis demonstrates that oxygen is a powerful regulator of bone cell formation and activity. Reduced oxygen tension stimulates osteoclastogenesis and has a reciprocal, inhibitory effect on osteoblast formation and activity. Hypoxia also causes acidosis, which would exacerbate the detrimental effects of hypoxia on bone. My studies may help to explain the bone loss associated with inflammation, fracture, tumour, diabetes, anaemia, ageing and infection; they also offer additional explanations for processes such as tooth movement, and provide new insights into the potential actions of vasoactive substances on bone. The vasculature is essential for provision of oxygen to the bone, and its role in regulating bone mass offers potential new therapeutic targets.

# Reference list

Aigner, T. and Stove, J. (2003) Collagens--major component of the physiological cartilage matrix, major target of cartilage degeneration, major tool in cartilage repair. *Adv Drug Deliv Rev*, **55**, 1569-1593.

Agocha, A., Lee, H.W. and Eghbali-Webb, M. (1997) Hypoxia regulates basal and induced DNA synthesis and collagen type I production in human cardiac fibroblasts: effects of transforming growth factor-beta1, thyroid hormone, angiotensin II and basic fibroblast growth factor. *J Mol Cell Cardiol*, **29**, 2233-2244.

Akeno, N., Czyzyk-Krzeska, M.F., Gross, T.S. and Clemens, T.L. (2001) Hypoxia induces vascular endothelial growth factor gene transcription in human osteoblast-like cells through the hypoxia-inducible factor-2 $\alpha$ . *Endocrinology*, **142**, 959-962.

Akeno, N., Robins, J., Zhang, M., Czyzyk-Krzeska, M.F. and Clemens, T.L. (2002) Induction of vascular endothelial growth factor by IGF-I in osteoblast-like cells is mediated by the PI3K signaling pathway through the hypoxia-inducible factor-2 $\alpha$ . *Endocrinology*, **143**, 420-425.

Ala, Y., Palluy, O., Favero, J., Bonne, C., Modat, G. and Dornand, J. (1992) Hypoxia/reoxygenation stimulates endothelial cells to promote interleukin-1 and interleukin-6 production. Effects of free radical scavengers. *Agents Actions*, **37**, 134-139.

Aldridge, S.E., Lennard, T.W., Williams, J.R. and Birch, M.A. (2005) Vascular endothelial growth factor receptors in osteoclast differentiation and function. *Biochem Biophys Res Commun*, **335**, 793-798.

Allen, C.B., Schneider, B.K. and White, C.W. (2001) Limitations to oxygen diffusion and equilibration in in vitro cell exposure systems in hyperoxia and hypoxia. *Am J Physiol Lung Cell Mol Physiol*, **281**, L1021-1027.



Allen, S.H., Nuttleman, P.R., Ketcham, C.M. and Roberts, R.M. (1989) Purification and characterization of human bone tartrate-resistant acid phosphatase. *J Bone Miner Res*, **4**, 47-55.

Almeida, M., Han, L., Bellido, T., Manolagas, S.C. and Kousteni, S. (2005) Wnts prevent apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblasts by beta -catenin-dependent as well as independent signaling cascades involving Src/ERK and PI3K/Akt. *J Biol Chem*, (*in press*).

Ambrosini, G., Nath, A.K., Sierra-Honigmann, M.R. and Flores-Riveros, J. (2002) Transcriptional activation of the human leptin gene in response to hypoxia. Involvement of hypoxia-inducible factor 1. *J Biol Chem*, **277**, 34601-34609.

Anderson, D.M., Maraskovsky, E., Billingsley, W.L., Dougall, W.C., Tometsko, M.E., Roux, E.R., Teepe, M.C., DuBose, R.F., Cosman, D. and Galibert, L. (1997) A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature*, **390**, 175-179.

Armour, K.E., Armour, K.J., Gallagher, M.E., Godecke, A., Helfrich, M.H., Reid, D.M. and Ralston, S.H. (2001) Defective bone formation and anabolic response to exogenous estrogen in mice with targeted disruption of endothelial nitric oxide synthase. *Endocrinology*, **142**, 760-766.

Arnett, T. (2003) Regulation of bone cell function by acid-base balance. *Proc Nutr Soc*, **62**, 511-520.

Arnett, T.R., Boyde, A., Jones, S.J. and Taylor, M.L. (1994) Effects of medium acidification by alteration of carbon dioxide or bicarbonate concentrations on the resorptive activity of rat osteoclasts. *J Bone Miner Res*, **9**, 375-379.

Arnett, T.R. and Dempster, D.W. (1986) Effect of pH on bone resorption by rat osteoclasts in vitro. *Endocrinology*, **119**, 119-124.

Arnett, T.R. and Dempster, D.W. (1987) A comparative study of disaggregated chick and rat osteoclasts in vitro: effects of calcitonin and prostaglandins. *Endocrinology*, **120**, 602-608.

Arnett, T.R., Gibbons, D.C., Utting, J.C., Orriss, I.R., Hoebertz, A., Rosendaal, M. and Meghji, S. (2003) Hypoxia is a major stimulator of osteoclast formation and bone resorption. *J Cell Physiol*, **196**, 2-8.

Arnett, T.R., Lindsay, R., Kilb, J.M., Moonga, B.S., Spowage, M. and Dempster, D.W. (1996) Selective toxic effects of tamoxifen on osteoclasts: comparison with the effects of oestrogen. *J Endocrinol*, **149**, 503-508.

Arnett, T.R. and Spowage, M. (1996) Modulation of the resorptive activity of rat osteoclasts by small changes in extracellular pH near the physiological range. *Bone*, **18**, 277-279.

Aubin, J.E., Liu, F., Malaval, L. and Gupta, A.K. (1995) Osteoblast and chondroblast differentiation. *Bone*, **17**, 77S-83S.

Bailey, A.J., Paul, R.G. and Knott, L. (1998) Mechanisms of maturation and ageing of collagen. *Mech Ageing Dev*, **106**, 1-56.

Bajayo, A., Goshen, I., Feldman, S., Csernus, V., Iverfeldt, K., Shohami, E., Yirmiya, R. and Bab, I. (2005) Central IL-1 receptor signaling regulates bone growth and mass. *Proc Natl Acad Sci U S A*, **102**, 12956-12961.

Balcerzak, M., Hamade, E., Zhang, L., Pikula, S., Azzar, G., Radisson, J., Bendorowicz-Pikula, J. and Buchet, R. (2003) The roles of annexins and alkaline phosphatase in mineralization process. *Acta Biochim Pol*, **50**, 1019-1038.

Barengolts, E.I., Berman, M., Kukreja, S.C., Kouznetsova, T., Lin, C. and Chomka, E.V. (1998) Osteoporosis and coronary atherosclerosis in asymptomatic postmenopausal women. *Calcif Tissue Int*, **62**, 209-213.

Barrett, M.G., Belinsky, G.S. and Tashjian, A.H., Jr. (1997) A new action of parathyroid hormone. receptor-mediated stimulation of extracellular acidification in human osteoblast-like SaOS-2 cells. *J Biol Chem*, **272**, 26346-26353.

Bellows, C.G., Aubin, J.E., Heersche, J.N. and Antosz, M.E. (1986) Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. *Calcif Tissue Int*, **38**, 143-154.

Berse, B., Hunt, J.A., Diegel, R.J., Morganelli, P., Yeo, K., Brown, F. and Fava, R.A. (1999) Hypoxia augments cytokine (transforming growth factor- $\beta$  (TGF- $\beta$ ) and IL-1)-induced vascular endothelial growth factor secretion by human synovial fibroblasts. *Clin Exp Immunol*, **115**, 176-182.

Bertolini, F., Mancuso, P., Gobbi, A. and Pruneri, G. (2000) The thin red line: angiogenesis in normal and malignant hematopoiesis. *Exp Hematol*, **28**, 993-1000.

Blair, H.C., Robinson, L.J. and Zaidi, M. (2005) Osteoclast signalling pathways. *Biochem Biophys Res Commun*, **328**, 728-738.

Bonnelye, E. and Aubin, J.E. (2002) Differential expression of estrogen receptor-related receptor  $\alpha$  and estrogen receptors  $\alpha$  and  $\beta$  in osteoblasts in vivo and in vitro. *J Bone Miner Res*, **17**, 1392-1400.

Borcsok, I., Schairer, H.U., Sommer, U., Wakley, G.K., Schneider, U., Geiger, F., Niethard, F.U., Ziegler, R. and Kasperk, C.H. (1998) Glucocorticoids regulate the expression of the human osteoblastic endothelin A receptor gene. *J Exp Med*, **188**, 1563-1573.

Bord, S., Ireland, D.C., Beavan, S.R. and Compston, J.E. (2003) The effects of estrogen on osteoprotegerin, RANKL, and estrogen receptor expression in human osteoblasts. *Bone*, **32**, 136-141.

Bos, R., van Diest, P.J., de Jong, J.S., van der Groep, P., van der Valk, P. and van der Wall, E. (2005) Hypoxia-inducible factor-1 $\alpha$  is associated with angiogenesis, and expression of bFGF, PDGF-BB, and EGFR in invasive breast cancer. *Histopathology*, **46**, 31-36.

Bosco, M.C., Puppo, M., Pastorino, S., Mi, Z., Melillo, G., Massazza, S., Rapisarda, A. and Varesio, L. (2004) Hypoxia selectively inhibits monocyte chemoattractant protein-1 production by macrophages. *J Immunol*, **172**, 1681-1690.

Bouachour, G., Cronier, P., Gouello, J.P., Toulemonde, J.L., Talha, A. and Alquier, P. (1996) Hyperbaric oxygen therapy in the management of crush injuries: a randomized double-blind placebo-controlled clinical trial. *J Trauma*, **41**, 333-339.

Bouletreau, P.J., Warren, S.M., Spector, J.A., Steinbrech, D.S., Mehrara, B.J. and Longaker, M.T. (2002) Factors in the fracture microenvironment induce primary osteoblast angiogenic cytokine production. *Plast Reconstr Surg*, **110**, 139-148.

Boyde, A. (2003) The real response of bone to exercise. *J Anat*, **203**, 173-189.

Boyde, A., Ali, N.N. and Jones, S.J. (1984) Resorption of dentine by isolated osteoclasts in vitro. *Br Dent J*, **156**, 216-220.

Bracken, C.P., Whitelaw, M.L. and Peet, D.J. (2005) Activity of hypoxia-inducible Factor 2 $\alpha$  is regulated by association with the NF- $\kappa$ B essential modulator. *J Biol Chem*, **280**, 14240-14251.

Bradley, T.R., Hodgson, G.S. and Rosendaal, M. (1978) The effect of oxygen tension on haemopoietic and fibroblast cell proliferation in vitro. *J Cell Physiol*, **97**, 517-522.

Brandao-Burch, A. and Arnett, T.R. (2004) Normal human osteoclasts are activated by acidosis. *J Bone Miner Res*, **19**, 1034 (abstract).

Brandao-Burch, A., Utting, J.C., Orriss, I.R. and Arnett, T.R. (2005) Acidosis inhibits bone formation by osteoblasts in vitro by preventing mineralization. *Calcif Tissue Int*, **77**, 167-174.

Bridgeman, G. and Brookes, M. (1996) Blood supply to the human femoral diaphysis in youth and senescence. *J Anat*, **188**, 611-621.

Brighton, C.T. and Krebs, A.G. (1972) Oxygen tension of healing fractures in the rabbit. *J Bone Joint Surg Am*, **54**, 323-332.

Brinckmann, J., Notbohm, H., Tronnier, M., Acil, Y., Fietzek, P.P., Schmeller, W., Muller, P.K. and Batge, B. (1999) Overhydroxylation of lysyl residues is the

initial step for altered collagen cross-links and fibril architecture in fibrotic skin. *J Invest Dermatol*, **113**, 617-621.

Brookes, M. and Revell, W.J. (1998) *Blood supply of bone: scientific aspects*. Springer, London; New York.

Browner, W.S., Seeley, D.G., Vogt, T.M. and Cummings, S.R. (1991) Non-trauma mortality in elderly women with low bone mineral density. Study of Osteoporotic Fractures Research Group. *Lancet*, **338**, 355-358.

Bruckdorfer, R. (2005) The basics about nitric oxide. *Mol Aspects Med*, **26**, 3-31.

Bucay, N., Sarosi, I., Dunstan, C.R., Morony, S., Tarpley, J., Capparelli, C., Scully, S., Tan, H.L., Xu, W., Lacey, D.L., Boyle, W.J. and Simonet, W.S. (1998) Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev*, **12**, 1260-1268.

Burgess, T.L., Qian, Y., Kaufman, S., Ring, B.D., Van, G., Capparelli, C., Kelley, M., Hsu, H., Boyle, W.J., Dunstan, C.R., Hu, S. and Lacey, D.L. (1999) The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *J Cell Biol*, **145**, 527-538.

Burnstock, G. (1987) Local control of blood pressure by purines. *Blood Vessels*, **24**, 156-160.

Callapina, M., Zhou, J., Schnitzer, S., Metzen, E., Lohr, C., Deitmer, J.W. and Brune, B. (2005) Nitric oxide reverses desferrioxamine- and hypoxia-evoked HIF-1 $\alpha$  accumulation-Implications for prolyl hydroxylase activity and iron. *Exp Cell Res*, **306**, 274-284.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R., Milner, L.A., Kronenberg, H.M. and Scadden, D.T. (2003) Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*, **425**, 841-846.

Carlevaro, M.F., Cermelli, S., Cancedda, R. and Descalzi, C.F. (2000) Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. *J Cell Sci*, **113**, 59-69.

Catrina, S.B., Okamoto, K., Pereira, T., Brismar, K. and Poellinger, L. (2004) Hyperglycemia regulates hypoxia-inducible factor-1 $\alpha$  protein stability and function. *Diabetes*, **53**, 3226-3232.

Cecchini, M.G., Hofstetter, W., Halasy, J., Wetterwald, A. and Felix, R. (1997) Role of CSF-1 in bone and bone marrow development. *Mol Reprod Dev*, **46**, 75-83; discussion 83-74.

Cernanec, J., Guilak, F., Weinberg, J.B., Pisetsky, D.S. and Fermor, B. (2002) Influence of hypoxia and reoxygenation on cytokine-induced production of proinflammatory mediators in articular cartilage. *Arthritis Rheum*, **46**, 968-975.

Chambers, T.J., Fuller, K., McSheehy, P.M. and Pringle, J.A. (1985) The effects of calcium regulating hormones on bone resorption by isolated human osteoclastoma cells. *J Pathol*, **145**, 297-305.

Chambers, T.J., Revell, P.A., Fuller, K. and Athanasou, N.A. (1984) Resorption of bone by isolated rabbit osteoclasts. *J Cell Sci*, **66**, 383-399.

Chambliss, K.L. and Shaul, P.W. (2002) Estrogen modulation of endothelial nitric oxide synthase. *Endocr Rev*, **23**, 665-686.

Cheng, B., Kato, Y., Zhao, S., Luo, J., Sprague, E., Bonewald, L.F. and Jiang, J.X. (2001) PGE<sub>2</sub> is essential for gap junction-mediated intercellular communication between osteocyte-like MLO-Y4 cells in response to mechanical strain. *Endocrinology*, **142**, 3464-3473.

Chow, D.C., Wenning, L.A., Miller, W.M. and Papoutsakis, E.T. (2001) Modeling pO<sub>2</sub> distributions in the bone marrow hematopoietic compartment. II. Modified Kroghian models. *Biophys J*, **81**, 685-696.

Chung, C.H., Golub, E.E., Forbes, E., Tokuoka, T. and Shapiro, I.M. (1992) Mechanism of action of beta-glycerophosphate on bone cell mineralization. *Calcif Tissue Int*, **51**, 305-311.

Clavel, G., Bessis, N. and Boissier, M.C. (2003) Recent data on the role for angiogenesis in rheumatoid arthritis. *Joint Bone Spine*, **70**, 321-326.

Cockman, M.E., Masson, N., Mole, D.R., Jaakkola, P., Chang, G.W., Clifford, S.C., Maher, E.R., Pugh, C.W., Ratcliffe, P.J. and Maxwell, P.H. (2000) Hypoxia inducible factor- $\alpha$  binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J Biol Chem*, **275**, 25733-25741.

Coimbra, I.B., Jimenez, S.A., Hawkins, D.F., Piera-Velazquez, S. and Stokes, D.G. (2004) Hypoxia inducible factor-1  $\alpha$  expression in human normal and osteoarthritic chondrocytes. *Osteoarthritis Cartilage*, **12**, 336-345.

Colleran, P.N., Wilkerson, M.K., Bloomfield, S.A., Suva, L.J., Turner, R.T. and Delp, M.D. (2000) Alterations in skeletal perfusion with simulated microgravity: a possible mechanism for bone remodeling. *J Appl Physiol*, **89**, 1046-1054.

Collin-Osdoby, P. (2004) Regulation of vascular calcification by osteoclast regulatory factors RANKL and osteoprotegerin. *Circ Res*, **95**, 1046-1057.

Collin-Osdoby, P., Rothe, L., Anderson, F., Nelson, M., Maloney, W. and Osdoby, P. (2001) Receptor activator of NF- $\kappa$ B and osteoprotegerin expression by human microvascular endothelial cells, regulation by inflammatory cytokines, and role in human osteoclastogenesis. *J Biol Chem*, **276**, 20659-20672.

Collin-Osdoby, P., Yu, X., Zheng, H. and Osdoby, P. (2003) RANKL-mediated osteoclast formation from murine RAW 264.7 cells. *Methods Mol Med*, **80**, 153-166.

Collins, D.A. and Chambers, T.J. (1991) Effect of prostaglandins E<sub>1</sub>, E<sub>2</sub>, and F<sub>2</sub>  $\alpha$  on osteoclast formation in mouse bone marrow cultures. *J Bone Miner Res*, **6**, 157-164.

Cornish, J., Callon, K.E., Bava, U., Lin, C., Naot, D., Hill, B.L., Grey, A.B., Broom, N., Myers, D.E., Nicholson, G.C. and Reid, I.R. (2002) Leptin directly regulates bone cell function *in vitro* and reduces bone fragility *in vivo*. *J Endocrinol*, **175**, 405-415.

Cornish, J., Callon, K.E., Cooper, G.J. and Reid, I.R. (1995) Amylin stimulates osteoblast proliferation and increases mineralized bone volume in adult mice. *Biochem Biophys Res Commun*, **207**, 133-139.

Cornish, J., Callon, K.E., King, A.R., Cooper, G.J. and Reid, I.R. (1998) Systemic administration of amylin increases bone mass, linear growth, and adiposity in adult male mice. *Am J Physiol*, **275**, E694-699.

Counts, D.F., Cardinale, G.J. and Udenfriend, S. (1978) Prolyl hydroxylase half reaction: peptidyl prolyl-independent decarboxylation of  $\alpha$ -ketoglutarate. *Proc Natl Acad Sci U S A*, **75**, 2145-2149.

Cowden Dahl, K.D., Robertson, S.E., Weaver, V.M. and Simon, M.C. (2005) Hypoxia-inducible factor regulates  $\alpha_v\beta_3$  integrin cell surface expression. *Mol Biol Cell*, **16**, 1901-1912.

Crabtree, G.R. and Olson, E.N. (2002) NFAT signaling: choreographing the social lives of cells. *Cell*, **109 Suppl**, S67-79.

Cramer, T., Yamanishi, Y., Clausen, B.E., Forster, I., Pawlinski, R., Mackman, N., Haase, V.H., Jaenisch, R., Corr, M., Nizet, V., Firestein, G.S., Gerber, H.P., Ferrara, N. and Johnson, R.S. (2003) HIF-1 $\alpha$  is essential for myeloid cell-mediated inflammation. *Cell*, **112**, 645-657.

Cui, W., Zhang Ke, J., Zhang, Q., Ke, H.Z., Chalouni, C. and Vignery, A. (2005) The intracellular domain of CD44 promotes the fusion of macrophages via NF $\kappa$ B. *Blood*, (in press).

Danet, G.H., Pan, Y., Luongo, J.L., Bonnet, D.A. and Simon, M.C. (2003) Expansion of human SCID-repopulating cells under hypoxic conditions. *J Clin Invest*, **112**, 126-135.



Day, C.J., Kim, M.S., Stephens, S.R., Simcock, W.E., Aitken, C.J., Nicholson, G.C. and Morrison, N.A. (2004) Gene array identification of osteoclast genes: differential inhibition of osteoclastogenesis by cyclosporin A and granulocyte macrophage colony stimulating factor. *J Cell Biochem*, **91**, 303-315.

de la Fuente, L. and Helms, J.A. (2005) Head, shoulders, knees, and toes. *Dev Biol*, **282**, 294-306.

de Vries, F., van Staa, T.P., Bracke, M.S., Cooper, C., Leufkens, H.G. and Lammers, J.W. (2005) Severity of obstructive airway disease and risk of osteoporotic fracture. *Eur Respir J*, **25**, 879-884.

Deckers, M.M., Karperien, M., van der, B.C., Yamashita, T., Papapoulos, S.E. and Lowik, C.W. (2000) Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. *Endocrinology*, **141**, 1667-1674.

Dempster, D.W. (1989) Bone histomorphometry in glucocorticoid-induced osteoporosis. *J Bone Miner Res*, **4**, 137-141.

Dempster, D.W., Hughes-Begos, C.E., Plavetic-Chee, K., Brandao-Burch, A., Cosman, F., Nieves, J., Neubort, S., Lu, S.S., Iida-Klein, A., Arnett, T. and Lindsay, R. (2005) Normal human osteoclasts formed from peripheral blood monocytes express PTH type 1 receptors and are stimulated by PTH in the absence of osteoblasts. *J Cell Biochem*, **95**, 139-148.

Denley, A., Cosgrove, L.J., Booker, G.W., Wallace, J.C. and Forbes, B.E. (2005) Molecular interactions of the IGF system. *Cytokine Growth Factor Rev*, **16**, 421-439.

Dery, M.A., Michaud, M.D. and Richard, D.E. (2005) Hypoxia-inducible factor 1: regulation by hypoxic and non-hypoxic activators. *Int J Biochem Cell Biol*, **37**, 535-540.

Dodd, J.S., Raleigh, J.A. and Gross, T.S. (1999) Osteocyte hypoxia: a novel mechanotransduction pathway. *Am J Physiol*, **277**, C598-C602.

Domm, C., Schunke, M., Christesen, K. and Kurz, B. (2002) Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension. *Osteoarthritis Cartilage*, **10**, 13-22.

Ducy, P., Amling, M., Takeda, S., Priemel, M., Schilling, A.F., Beil, F.T., Shen, J., Vinson, C., Rueger, J.M. and Karsenty, G. (2000) Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell*, **100**, 197-207.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A.L. and Karsenty, G. (1997) *Osx2/Cbfa1*: a transcriptional activator of osteoblast differentiation. *Cell*, **89**, 747-754.

Dupont, J. and Holzenberger, M. (2003) Biology of insulin-like growth factors in development. *Birth Defects Res C Embryo Today*, **69**, 257-271.

Eames, B.F., Sharpe, P.T. and Helms, J.A. (2004) Hierarchy revealed in the specification of three skeletal fates by *Sox9* and *Runx2*. *Dev Biol*, **274**, 188-200.

Eghbali-Fatourehchi, G.Z., Lamsam, J., Fraser, D., Nagel, D., Riggs, B.L. and Khosla, S. (2005) Circulating osteoblast-lineage cells in humans. *N Engl J Med*, **352**, 1959-1966.

Einhorn, T.A. (1998) The cell and molecular biology of fracture healing. *Clin Orthop Relat Res*, **355 (Sup)**, S7-21.

Emerling, B.M., Plataniias, L.C., Black, E., Nebreda, A.R., Davis, R.J. and Chandel, N.S. (2005) Mitochondrial reactive oxygen species activation of p38 mitogen-activated protein kinase is required for hypoxia signaling. *Mol Cell Biol*, **25**, 4853-4862.

Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A., Tian, Y.M., Masson, N., Hamilton, D.L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P.H., Pugh, C.W., Schofield, C.J. and Ratcliffe, P.J. (2001) *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell*, **107**, 43-54.

Estabrook, R.W. (2005) Steroid hydroxylations: a paradigm for cytochrome P450 catalyzed mammalian monooxygenation reactions. *Biochem Biophys Res Commun*, **338**, 290-298.

Faglia, E., Favales, F., Quarantiello, A., Calia, P., Clelia, P., Brambilla, G., Rampoldi, A. and Morabito, A. (1998) Angiographic evaluation of peripheral arterial occlusive disease and its role as a prognostic determinant for major amputation in diabetic subjects with foot ulcers. *Diabetes Care*, **21**, 625-630.

Falanga, V., Zhou, L. and Yufit, T. (2002) Low oxygen tension stimulates collagen synthesis and COL1A1 transcription through the action of TGF- $\beta$ 1. *J Cell Physiol*, **191**, 42-50.

Fandrey, J. (1995) Hypoxia-inducible gene expression. *Respir Physiol*, **101**, 1-10.

Feng, X. (2005) Regulatory roles and molecular signaling of TNF family members in osteoclasts. *Gene*, **350**, 1-13.

Fessler, L.I. and Fessler, J.H. (1974) Protein assembly of procollagen and effects of hydroxylation. *J Biol Chem*, **249**, 7637-7646.

Fink, T., Abildtrup, L., Fogd, K., Abdallah, B.M., Kassem, M., Ebbesen, P. and Zachar, V. (2004) Induction of adipocyte-like phenotype in human mesenchymal stem cells by hypoxia. *Stem Cells*, **22**, 1346-1355.

Firestein, G.S. (2003) Evolving concepts of rheumatoid arthritis. *Nature*, **423**, 356-361.

Flanagan, A.M., Horton, M.A., Dorey, E.L., Collins, D.A., Evely, R.S., Moseley, J.M., Firkin, F.C., Chambers, T.J., Helfrich, M.H. and Martin, T.J. (1992) An assessment of the ability of human bone marrow cultures to generate osteoclasts. *Int J Exp Pathol*, **73**, 387-401.

Flick, L.M., Weaver, J.M., Ulrich-Vinther, M., Abuzzahab, F., Zhang, X., Dougall, W.C., Anderson, D., O'Keefe, R.J. and Schwarz, E.M. (2003) Effects of

receptor activator of NF $\kappa$ B (RANK) signaling blockade on fracture healing. *J Orthop Res*, **21**, 676-684.

Franciosi, L.G., Page, C.P., Celli, B.R., Cazzola, M., Walker, M.J., Danhof, M., Rabe, K.F. and Pasqua, O.E. (2005) Markers of disease severity in chronic obstructive pulmonary disease. *Pulm Pharmacol Ther.* (In press).

Franzoso, G., Carlson, L., Xing, L., Poljak, L., Shores, E.W., Brown, K.D., Leonardi, A., Tran, T., Boyce, B.F. and Siebenlist, U. (1997) Requirement for NF- $\kappa$ B in osteoclast and B-cell development. *Genes Dev*, **11**, 3482-3496.

Fraser, J.H., Helfrich, M.H., Wallace, H.M. and Ralston, S.H. (1996) Hydrogen peroxide, but not superoxide, stimulates bone resorption in mouse calvariae. *Bone*, **19**, 223-226.

Frost, H.M. (1998) A brief review for orthopedic surgeons: fatigue damage (microdamage) in bone (its determinants and clinical implications). *J Orthop Sci*, **3**, 272-281.

Fukuoka, H., Aoyama, M., Miyazawa, K., Asai, K. and Goto, S. (2005) Hypoxic stress enhances osteoclast differentiation via increasing IGF2 production by non-osteoclastic cells. *Biochem Biophys Res Commun*, **328**, 885-894.

Fukushima, N., Hanada, R., Teranishi, H., Fukue, Y., Tachibana, T., Ishikawa, H., Takeda, S., Takeuchi, Y., Fukumoto, S., Kangawa, K., Nagata, K. and Kojima, M. (2005) Ghrelin directly regulates bone formation. *J Bone Miner Res*, **20**, 790-798.

Fuller, K., Lean, J.M., Bayley, K.E., Wani, M.R. and Chambers, T.J. (2000) A role for TGF $\beta$ <sub>1</sub> in osteoclast differentiation and survival. *J Cell Sci*, **113**, 2445.

Fuller, K., Wong, B., Fox, S., Choi, Y. and Chambers, T.J. (1998) TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. *J Exp Med*, **188**, 997-1001.

Gallez, B. and Mader, K. (2000) Accurate and sensitive measurements of pO<sub>2</sub> in vivo using low frequency EPR spectroscopy: how to confer biocompatibility to the oxygen sensors. *Free Radic Biol Med*, **29**, 1078-1084.

Garnero, P., Borel, O., Byrjalsen, I., Ferreras, M., Drake, F.H., McQueney, M.S., Foged, N.T., Delmas, P.D. and Delaisse, J.M. (1998) The collagenolytic activity of cathepsin K is unique among mammalian proteinases. *J Biol Chem*, **273**, 32347-32352.

Garrett, I.R., Boyce, B.F., Oreffo, R.O., Bonewald, L., Poser, J. and Mundy, G.R. (1990) Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. *J Clin Invest*, **85**, 632-639.

Gaur, T., Lengner, C.J., Hovhannisyan, H., Bhat, R.A., Bodine, P.V., Komm, B.S., Javed, A., van Wijnen, A.J., Stein, J.L., Stein, G.S. and Lian, J.B. (2005) Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *J Biol Chem*, **280**, 33132-33140.

Gersch, R.P., Lombardo, F., McGovern, S.C. and Hadjiargyrou, M. (2005) Reactivation of Hox gene expression during bone regeneration. *J Orthop Res*, **23**, 882-890.

Giatromanolaki, A., Sivridis, E., Maltezos, E., Athanassou, N., Papazoglou, D., Gatter, K.C., Harris, A.L. and Koukourakis, M.I. (2003) Upregulated hypoxia inducible factor-1 $\alpha$  and -2 $\alpha$  pathway in rheumatoid arthritis and osteoarthritis. *Arthritis Res Ther*, **5**, R193-201.

Goldhaber, P. and Rabadjija, L. (1987) H<sup>+</sup> stimulation of cell-mediated bone resorption in tissue culture. *Am J Physiol*, **253**, E90-98.

Gong, Y., Slee, R.B., Fukai, N., Rawadi, G., Roman-Roman, S., Reginato, A.M., Wang, H., Cundy, T., Glorieux, F.H., Lev, D., Zacharin, M., Oexle, K., Marcelino, J., Suwairi, W., Heeger, S., Sabatakos, G., Apte, S., Adkins, W.N., Allgrove, J., Arslan-Kirchner, M., Batch, J.A., Beighton, P., Black, G.C., Boles, R.G., Boon, L.M., Borrone, C., Brunner, H.G., Carle, G.F., Dallapiccola, B., De Paepe, A., Floege, B., Halfhide, M.L., Hall, B., Hennekam, R.C., Hirose, T., Jans, A., Juppner, H., Kim,

C.A., Keppler-Noreuil, K., Kohlschuetter, A., LaCombe, D., Lambert, M., Lemyre, E., Letteboer, T., Peltonen, L., Ramesar, R.S., Romanengo, M., Somer, H., Steichen-Gersdorf, E., Steinmann, B., Sullivan, B., Superti-Furga, A., Swoboda, W., van den Boogaard, M.J., Van Hul, W., Vikkula, M., Votruba, M., Zabel, B., Garcia, T., Baron, R., Olsen, B.R. and Warman, M.L. (2001) LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell*, **107**, 513-523.

Gordeladze, J.O., Drevon, C.A., Syversen, U. and Reseland, J.E. (2002) Leptin stimulates human osteoblastic cell proliferation, de novo collagen synthesis, and mineralization: Impact on differentiation markers, apoptosis, and osteoclastic signaling. *J Cell Biochem*, **85**, 825-836.

Gorny, G., Shaw, A. and Oursler, M.J. (2004) IL-6, LIF, and TNF- $\alpha$  regulation of GM-CSF inhibition of osteoclastogenesis in vitro. *Exp Cell Res*, **294**, 149-158.

Goto, T., Yamaza, T. and Tanaka, T. (2003) Cathepsins in the osteoclast. *J Electron Microsc (Tokyo)*, **52**, 551-558.

Grano, M., Mori, G., Minielli, V., Barou, O., Colucci, S., Giannelli, G., Alexandre, C., Zallone, A.Z. and Vico, L. (2002) Rat hindlimb unloading by tail suspension reduces osteoblast differentiation, induces IL-6 secretion, and increases bone resorption in *ex vivo* cultures. *Calcif Tissue Int*, **70**, 176-185.

Gray, C., Colston, K.W., Mackay, A.G., Taylor, M.L. and Arnett, T.R. (1992) Interaction of androgen and 1,25-dihydroxyvitamin D3: effects on normal rat bone cells. *J Bone Miner Res*, **7**, 41-46.

Grigoriadis, A.E., Heersche, J.N. and Aubin, J.E. (1988) Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. *J Cell Biol*, **106**, 2139-2151.

Grigoriadis, A.E., Wang, Z.Q., Cecchini, M.G., Hofstetter, W., Felix, R., Fleisch, H.A. and Wagner, E.F. (1994) c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science*, **266**, 443-448.

Grimshaw, M.J. and Mason, R.M. (2000) Bovine articular chondrocyte function in vitro depends upon oxygen tension. *Osteoarthritis Cartilage*, **8**, 386-392.

Grosfeld, A., Zilberfarb, V., Turban, S., Andre, J., Guerre-Millo, M. and Issad, T. (2002) Hypoxia increases leptin expression in human PAZ6 adipose cells. *Diabetologia*, **45**, 527-530.

Gross, T.S., Akeno, N., Clemens, T.L., Komarova, S., Srinivasan, S., Weimer, D.A. and Mayorov, S. (2001) Selected Contribution: Osteocytes upregulate HIF-1 $\alpha$  in response to acute disuse and oxygen deprivation. *J Appl Physiol*, **90**, 2514-2519.

Gu, G., Mulari, M., Peng, Z., Hentunen, T.A. and Vaananen, H.K. (2005) Death of osteocytes turns off the inhibition of osteoclasts and triggers local bone resorption. *Biochem Biophys Res Commun*, **335**, 1095-1101.

Haddad, J. (2002) Redox regulation of pro-inflammatory cytokines and I $\kappa$ B- $\alpha$ /NF- $\kappa$ B nuclear translocation and activation. *Biochem Biophys Res Commun*, **296**, 847-856.

Hamburger, V. and Hamilton, H.L. (1951) A series of normal stages in the development of the chick embryo. *J Morphol*, **88**, 49-92.

Hamersky, P.A., Weimer, A.D. and Taintor, J.F. (1980) The effect of orthodontic force application on the pulpal tissue respiration rate in the human premolar. *Am J Orthod*, **77**, 368-378.

Hara, S., Hamada, J., Kobayashi, C., Kondo, Y. and Imura, N. (2001) Expression and characterization of hypoxia-inducible factor (HIF)-3 $\alpha$  in human kidney: suppression of HIF-mediated gene expression by HIF-3 $\alpha$ . *Biochem Biophys Res Commun*, **287**, 808-813.

Harrison, J.S., Rameshwar, P., Chang, V. and Bandari, P. (2002) Oxygen saturation in the bone marrow of healthy volunteers. *Blood*, **99**, 394.

Hassan, M.Q., Javed, A., Morasso, M.I., Karlin, J., Montecino, M., van Wijnen, A.J., Stein, G.S., Stein, J.L. and Lian, J.B. (2004) Dlx3 transcriptional regulation of

osteoblast differentiation: temporal recruitment of Msx2, Dlx3, and Dlx5 homeodomain proteins to chromatin of the osteocalcin gene. *Mol Cell Biol*, **24**, 9248-9261.

Hattersley, G. and Chambers, T.J. (1989) Generation of osteoclasts from hemopoietic cells and a multipotential cell line in vitro. *J Cell Physiol*, **140**, 478-482.

Hayman, A.R., Jones, S.J., Boyde, A., Foster, D., Colledge, W.H., Carlton, M.B., Evans, M.J. and Cox, T.M. (1996) Mice lacking tartrate-resistant acid phosphatase (Acp 5) have disrupted endochondral ossification and mild osteopetrosis. *Development*, **122**, 3151-3162.

Helfrich, M.H., Nesbitt, S.A., Lakkakorpi, P.T., Barnes, M.J., Bodary, S.C., Shankar, G., Mason, W.T., Mendrick, D.L., Vaananen, H.K. and Horton, M.A. (1996)  $\beta 1$  integrins and osteoclast function: involvement in collagen recognition and bone resorption. *Bone*, **19**, 317-328.

Hellwig-Burgel, T., Stiehl, D.P., Wagner, A.E., Metzen, E. and Jelkmann, W. (2005) Review: hypoxia-inducible factor-1 (HIF-1): a novel transcription factor in immune reactions. *J Interferon Cytokine Res*, **25**, 297-310.

Herbertson, A. and Aubin, J.E. (1997) Cell sorting enriches osteogenic populations in rat bone marrow stromal cell cultures. *Bone*, **21**, 491-500.

Hershey, C.L. and Fisher, D.E. (2004) Mitf and Tfe3: members of a b-HLH-ZIP transcription factor family essential for osteoclast development and function. *Bone*, **34**, 689-696.

Hevehan, D.L., Papoutsakis, E.T. and Miller, W.M. (2000) Physiologically significant effects of pH and oxygen tension on granulopoiesis. *Exp Hematol*, **28**, 267-275.

Hoebertz, A. and Arnett, T.R. (2003) Isolated osteoclast cultures. *Methods Mol Med*, **80**, 53-64.



Hoebertz, A., Arnett, T.R. and Burnstock, G. (2003) Regulation of bone resorption and formation by purines and pyrimidines. *Trends Pharmacol Sci*, **24**, 290-297.

Hoebertz, A., Mahendran, S., Burnstock, G. and Arnett, T.R. (2002) ATP and UTP at low concentrations strongly inhibit bone formation by osteoblasts: a novel role for the P2Y<sub>2</sub> receptor in bone remodeling. *J Cell Biochem*, **86**, 413-419.

Hoebertz, A., Meghji, S., Burnstock, G. and Arnett, T.R. (2001) Extracellular ADP is a powerful osteolytic agent: evidence for signaling through the P2Y<sub>1</sub> receptor on bone cells. *Faseb J*, **15**, 1139-1148.

Hofbauer, K.H., Gess, B., Lohaus, C., Meyer, H.E., Katschinski, D. and Kurtz, A. (2003) Oxygen tension regulates the expression of a group of procollagen hydroxylases. *Eur J Biochem*, **270**, 4515-4522.

Hofbauer, L.C., Khosla, S., Dunstan, C.R., Lacey, D.L., Spelsberg, T.C. and Riggs, B.L. (1999) Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells. *Endocrinology*, **140**, 4367-4370.

Hollberg, K., Hultenby, K., Hayman, A., Cox, T. and Andersson, G. (2002) Osteoclasts from mice deficient in tartrate-resistant acid phosphatase have altered ruffled borders and disturbed intracellular vesicular transport. *Exp Cell Res*, **279**, 227-238.

Horton, M.A. (1995) Interactions of connective tissue cells with the extracellular matrix. *Bone*, **17**, 51S-53S.

Horton, M.A., Taylor, M.L., Arnett, T.R. and Helfrich, M.H. (1991) Arg-Gly-Asp (RGD) peptides and the anti-vitronectin receptor antibody 23C6 inhibit dentine resorption and cell spreading by osteoclasts. *Exp Cell Res*, **195**, 368-375.

Horwood, N.J., Kartsogiannis, V., Quinn, J.M., Romas, E., Martin, T.J. and Gillespie, M.T. (1999) Activated T lymphocytes support osteoclast formation *in vitro*. *Biochem Biophys Res Commun*, **265**, 144-150.

Hsu, H., Lacey, D.L., Dunstan, C.R., Solovyev, I., Colombero, A., Timms, E., Tan, H.L., Elliott, G., Kelley, M.J., Sarosi, I., Wang, L., Xia, X.Z., Elliott, R., Chiu, L., Black, T., Scully, S., Capparelli, C., Morony, S., Shimamoto, G., Bass, M.B. and Boyle, W.J. (1999) Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci U S A*, **96**, 3540-3545.

Hu, C.J., Wang, L.Y., Chodosh, L.A., Keith, B. and Simon, M.C. (2003) Differential roles of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) and HIF-2 $\alpha$  in hypoxic gene regulation. *Mol Cell Biol*, **23**, 9361-9374.

Huang, L.E., Arany, Z., Livingston, D.M. and Bunn, H.F. (1996) Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its  $\alpha$  subunit. *J Biol Chem*, **271**, 32253-32259.

Hunter, C., Barer, G.R., Shaw, J.W. and Clegg, E.J. (1974) Growth of the heart and lungs in hypoxic rodents: a model of human hypoxic disease. *Clin Sci Mol Med*, **46**, 375-391.

Iliopoulos, O., Levy, A.P., Jiang, C., Kaelin, W.G., Jr. and Goldberg, M.A. (1996) Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. *Proc Natl Acad Sci U S A*, **93**, 10595-10599.

Ishida, A., Fujita, N., Kitazawa, R. and Tsuruo, T. (2002) Transforming growth factor-beta induces expression of receptor activator of NF- $\kappa$ B ligand in vascular endothelial cells derived from bone. *J Biol Chem*, **277**, 26217-26224.

Ishikawa, Y. and Ito, T. (1988) Kinetics of hemopoietic stem cells in a hypoxic culture. *Eur J Haematol*, **40**, 126-129.

Ivanovic, Z., Bartolozzi, B., Bernabei, P.A., Cipolleschi, M.G., Rovida, E., Milenkovic, P., Praloran, V. and Dello Sbarba, P. (2000) Incubation of murine bone marrow cells in hypoxia ensures the maintenance of marrow-repopulating ability together with the expansion of committed progenitors. *Br J Haematol*, **108**, 424-429.

Ivanovic, Z., Belloc, F., Faucher, J.L., Cipolleschi, M.G., Praloran, V. and Dello, S.P. (2002) Hypoxia maintains and interleukin-3 reduces the pre-colony-forming cell potential of dividing CD34<sup>+</sup> murine bone marrow cells. *Exp Hematol*, **30**, 67-73.

Ivanovic, Z., Hermitte, F., de la Grange, P.B., Dazey, B., Belloc, F., Lacombe, F., Vezon, G. and Praloran, V. (2004) Simultaneous maintenance of human cord blood SCID-repopulating cells and expansion of committed progenitors at low O<sub>2</sub> concentration (3%). *Stem Cells*, **22**, 716-724.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y. and Semenza, G.L. (1998) Cellular and developmental control of O<sub>2</sub> homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev*, **12**, 149-162.

Jahr, H., van Driel, M., van Osch, G.J., Weinans, H. and van Leeuwen, J.P. (2005) Identification of acid-sensing ion channels in bone. *Biochem Biophys Res Commun*, **337**, 349-354.

Janssens, J.P., Pache, J.C. and Nicod, L.P. (1999) Physiological changes in respiratory function associated with ageing. *Eur Respir J*, **13**, 197-205.

Janssens, K., Ten Dijke, P., Janssens, S. and Van Hul, W. (2005) TGF- $\beta$ 1 to the bone. *Endocr Rev*, **26**, 743-774.

Johnson, C., Sung, H.J., Lessner, S.M., Fini, M.E. and Galis, Z.S. (2004) Matrix metalloproteinase-9 is required for adequate angiogenic revascularization of ischemic tissues: potential role in capillary branching. *Circ Res*, **94**, 262-268.

Kagan, H.M. and Li, W. (2003) Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. *J Cell Biochem*, **88**, 660-672.

Kanazawa, K. and Kudo, A. (2005) TRAF2 is essential for TNF- $\alpha$ -induced osteoclastogenesis. *J Bone Miner Res*, **20**, 840-847.

Kapur, S., Baylink, D.J. and William Lau, K.H. (2003) Fluid flow shear stress stimulates human osteoblast proliferation and differentiation through multiple interacting and competing signal transduction pathways. *Bone*, **32**, 241-251.

Kartsogiannis, V. and Ng, K.W. (2004) Cell lines and primary cell cultures in the study of bone cell biology. *Mol Cell Endocrinol*, **228**, 79-102.

Kenner, L., Hoebertz, A., Beil, T., Keon, N., Karreth, F., Eferl, R., Scheuch, H., Szremska, A., Amling, M., Schorpp-Kistner, M., Angel, P. and Wagner, E.F. (2004) Mice lacking JunB are osteopenic due to cell-autonomous osteoblast and osteoclast defects. *J Cell Biol*, **164**, 613-623.

Kerwin, S.C., Lewis, D.D., Elkins, A.D., Oliver, J.L., Hosgood, G., Pechman, R.D., Jr., Dial, S.L. and Strain, G.M. (2000) Effect of hyperbaric oxygen treatment on incorporation of an autogenous cancellous bone graft in a nonunion diaphyseal ulnar defect in cats. *Am J Vet Res*, **61**, 691-698.

Kietzmann, T. and Gorlach, A. (2005) Reactive oxygen species in the control of hypoxia-inducible factor-mediated gene expression. *Semin Cell Dev Biol*, (in press).

Kikuchi, Y., Suzuki, Y. and Tamiya, N. (1983) The source of oxygen in the reaction catalysed by collagen lysyl hydroxylase. *Biochem J*, **213**, 507-512.

Kim, H.H., Lee, S.E., Chung, W.J., Choi, Y., Kwack, K., Kim, S.W., Kim, M.S., Park, H. and Lee, Z.H. (2002) Stabilization of hypoxia-inducible factor-1 $\alpha$  is involved in the hypoxic stimuli-induced expression of vascular endothelial growth factor in osteoblastic cells. *Cytokine*, **17**, 14-27.

Kim, H.H., Shin, H.S., Kwak, H.J., Ahn, K.Y., Kim, J.H., Lee, H.J., Lee, M.S., Lee, Z.H. and Koh, G.Y. (2003) RANKL regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. *Faseb J*, **17**, 2163-2165.

Kim, K.H., Song, M.J., Chung, J., Park, H. and Kim, J.B. (2005) Hypoxia inhibits adipocyte differentiation in a HDAC-independent manner. *Biochem Biophys Res Commun*, **333**, 1178-1184.

Kiviranta, R., Morko, J., Alatalo, S.L., NicAmhlaoibh, R., Risteli, J., Laitala-Leinonen, T. and Vuorio, E. (2005) Impaired bone resorption in cathepsin K-deficient mice is partially compensated for by enhanced osteoclastogenesis and increased expression of other proteases via an increased RANKL/OPG ratio. *Bone*, **36**, 159-172.

Klein-Nulend, J., van der Plas, A., Semeins, C.M., Ajubi, N.E., Frangos, J.A., Nijweide, P.J. and Burger, E.H. (1995) Sensitivity of osteocytes to biomechanical stress *in vitro*. *Faseb J*, **9**, 441-445.

Knothe Tate, M.L., Adamson, J.R., Tami, A.E. and Bauer, T.W. (2004) The osteocyte. *Int J Biochem Cell Biol*, **36**, 1-8.

Knothe Tate, M.L. and Knothe, U. (2000) An *ex vivo* model to study transport processes and fluid flow in loaded bone. *J Biomech*, **33**, 247-254.

Knothe Tate, M.L., Niederer, P. and Knothe, U. (1998) In vivo tracer transport through the lacunocanalicular system of rat bone in an environment devoid of mechanical loading. *Bone*, **22**, 107-117.

Kobayashi, T. and Kronenberg, H. (2005) Minireview: transcriptional regulation in development of bone. *Endocrinology*, **146**, 1012-1017.

Koga, T., Matsui, Y., Asagiri, M., Kodama, T., de Crombrughe, B., Nakashima, K. and Takayanagi, H. (2005) NFAT and Osterix cooperatively regulate bone formation. *Nat Med*, **11**, 880-885.

Komatsu, D.E. and Hadjiargyrou, M. (2004) Activation of the transcription factor HIF-1 and its target genes, VEGF, HO-1, iNOS, during fracture repair. *Bone*, **34**, 680-688.

Komori, T. (2003) Requisite roles of Runx2 and Cbfb in skeletal development. *J Bone Miner Metab*, **21**, 193-197.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R.T., Gao, Y.H., Inada, M., Sato, M., Okamoto, R., Kitamura,

Y., Yoshiki, S. and Kishimoto, T. (1997) Targeted disruption of *Cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell*, **89**, 755-764.

Kong, Y.Y., Boyle, W.J. and Penninger, J.M. (1999) Osteoprotegerin ligand: a common link between osteoclastogenesis, lymph node formation and lymphocyte development. *Immunol Cell Biol*, **77**, 188-193.

Kong, Y.Y., Yoshida, H., Sarosi, I., Tan, H.L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A.J., Van, G., Itie, A., Khoo, W., Wakeham, A., Dunstan, C.R., Lacey, D.L., Mak, T.W., Boyle, W.J. and Penninger, J.M. (1999) OPG is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature*, **397**, 315-323.

Kotch, L.E., Iyer, N.V., Laughner, E. and Semenza, G.L. (1999) Defective vascularization of HIF-1 $\alpha$ -null embryos is not associated with VEGF deficiency but with mesenchymal cell death. *Dev Biol*, **209**, 254-267.

Krieger, N.S., Parker, W.R., Alexander, K.M. and Bushinsky, D.A. (2000) Prostaglandins regulate acid-induced cell-mediated bone resorption. *Am J Physiol Renal Physiol*, **279**, F1077-1082.

Kudo, O., Fujikawa, Y., Itonaga, I., Sabokbar, A., Torisu, T. and Athanasou, N.A. (2002) Proinflammatory cytokine (TNF $\alpha$ /IL-1 $\alpha$ ) induction of human osteoclast formation. *J Pathol*, **198**, 220-227.

Kudo, O., Sabokbar, A., Pocock, A., Itonaga, I., Fujikawa, Y. and Athanasou, N.A. (2003) Interleukin-6 and interleukin-11 support human osteoclast formation by a RANKL-independent mechanism. *Bone*, **32**, 1-7.

Kwan Tat, S., Padrines, M., Theoleyre, S., Heymann, D. and Fortun, Y. (2004) IL-6, RANKL, TNF- $\alpha$ /IL-1: interrelations in bone resorption pathophysiology. *Cytokine Growth Factor Rev*, **15**, 49-60.

Kwon, N.S., Nathan, C.F., Gilker, C., Griffith, O.W., Matthews, D.E. and Stuehr, D.J. (1990) L-citrulline production from L-arginine by macrophage nitric oxide synthase. The ureido oxygen derives from dioxygen. *J Biol Chem*, **265**, 13442-13445.

Kwon, O.H., Lee, C.K., Lee, Y.I., Paik, S.G. and Lee, H.J. (2005) The hematopoietic transcription factor PU.1 regulates RANK gene expression in myeloid progenitors. *Biochem Biophys Res Commun*, **335**, 437-446.

Lacey, D.L., Timms, E., Tan, H.L., Kelley, M.J., Dunstan, C.R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y.X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J. and Boyle, W.J. (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell*, **93**, 165-176.

Lader, C.S. and Flanagan, A.M. (1998) Prostaglandin E<sub>2</sub>, interleukin 1 $\alpha$ , and tumor necrosis factor- $\alpha$  increase human osteoclast formation and bone resorption in vitro. *Endocrinology*, **139**, 3157-3164.

Lafage-Proust, M.H., Boudignon, B. and Thomas, T. (2003) Glucocorticoid-induced osteoporosis: pathophysiological data and recent treatments. *Joint Bone Spine*, **70**, 109-118.

LaLuppa, J.A., Papoutsakis, E.T. and Miller, W.M. (1998) Oxygen tension alters the effects of cytokines on the megakaryocyte, erythrocyte, and granulocyte lineages. *Exp. Hematol.*, **26**, 835-843.

Lam, J., Takeshita, S., Barker, J.E., Kanagawa, O., Ross, F.P. and Teitelbaum, S.L. (2000) TNF- $\alpha$  induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J Clin Invest*, **106**, 1481-1488.

Lando, D., Peet, D.J., Whelan, D.A., Gorman, J.J. and Whitelaw, M.L. (2002) Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science*, **295**, 858-861.

Lands, W.E., Sauter, J. and Stone, G.W. (1978) Oxygen requirement for prostaglandin biosynthesis. *Prostaglandins Med*, **1**, 117-120.

Laroche, M. (2002) Intraosseous circulation from physiology to disease. *Joint Bone Spine*, **69**, 262-269.

Lattimore, J.D., Wilcox, I., Nakhla, S., Langenfeld, M., Jessup, W. and Celermajer, D.S. (2005) Repetitive hypoxia increases lipid loading in human macrophages-a potentially atherogenic effect. *Atherosclerosis*, **179**, 255-259.

Lean, J.M., Jagger, C.J., Kirstein, B., Fuller, K. and Chambers, T.J. (2005) Hydrogen peroxide is essential for estrogen-deficiency bone loss and osteoclast formation. *Endocrinology*, **146**, 728-735.

Lee, N.K., Choi, Y.G., Baik, J.Y., Han, S.Y., Jeong, D.W., Bae, Y.S., Kim, N. and Lee, S.Y. (2005) A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. *Blood*, **106**, 852-859.

Lee, S.E., Chung, W.J., Kwak, H.B., Chung, C.H., Kwack, K.B., Lee, Z.H. and Kim, H.H. (2001) Tumor necrosis factor- $\alpha$  supports the survival of osteoclasts through the activation of Akt and ERK. *J Biol Chem*, **276**, 49343-49349.

Lee, S.K., Gardner, A.E., Kalinowski, J.F., Jastrzebski, S.L. and Lorenzo, J.A. (2005) RANKL-stimulated osteoclast-like cell formation in vitro is partially dependent on endogenous interleukin-1 production. *Bone*, (*in press*).

Leek, R.D., Talks, K.L., Pezzella, F., Turley, H., Campo, L., Brown, N.S., Bicknell, R., Taylor, M., Gatter, K.C. and Harris, A.L. (2002) Relation of hypoxia-inducible factor-2  $\alpha$  (HIF-2  $\alpha$ ) expression in tumor-infiltrative macrophages to tumor angiogenesis and the oxidative thymidine phosphorylase pathway in human breast cancer. *Cancer Res*, **62**, 1326-1329.

Lennon, D.P., Edmison, J.M. and Caplan, A.I. (2001) Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on *in vitro* and *in vivo* osteochondrogenesis. *J Cell Physiol*, **187**, 345-355.

Lewis, C. and Murdoch, C. (2005) Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies. *Am J Pathol*, **167**, 627-635.



Lewis, J.S., Lee, J.A., Underwood, J.C., Harris, A.L. and Lewis, C.E. (1999) Macrophage responses to hypoxia: relevance to disease mechanisms. *J Leukoc Biol*, **66**, 889-900.

Liu, X.H., Kirschenbaum, A., Yao, S. and Levine, A.C. (2005) Cross-talk between the interleukin-6 and prostaglandin E<sub>2</sub> signaling systems results in enhancement of osteoclastogenesis through effects on the osteoprotegerin/receptor activator of nuclear factor- $\kappa$ B (RANK) ligand/RANK system. *Endocrinology*, **146**, 1991-1998.

Lomaga, M.A., Yeh, W.C., Sarosi, I., Duncan, G.S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G., Kaufman, S., van der Heiden, A., Itie, A., Wakeham, A., Khoo, W., Sasaki, T., Cao, Z., Penninger, J.M., Paige, C.J., Lacey, D.L., Dunstan, C.R., Boyle, W.J., Goeddel, D.V. and Mak, T.W. (1999) TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev*, **13**, 1015-1024.

Lopez-Barneo, J., del Toro, R., Levitsky, K.L., Chiara, M.D. and Ortega-Saenz, P. (2004) Regulation of oxygen sensing by ion channels. *J Appl Physiol*, **96**, 1187-1195; discussion 1170-1182.

Lowe, C., Yoneda, T., Boyce, B.F., Chen, H., Mundy, G.R. and Soriano, P. (1993) Osteopetrosis in Src-deficient mice is due to an autonomous defect of osteoclasts. *Proc Natl Acad Sci U S A*, **90**, 4485-4489.

Lu, H., Dalgard, C., Mohyeldin, A., McFate, T., Tait, A.S. and Verma, A. (2005) Reversible inactivation of HIF-1 prolyl hydroxylases allows cell metabolism to control basal HIF-1. *J Biol Chem*, (in press).

Ludwig, M.G., Vanek, M., Guerini, D., Gasser, J.A., Jones, C.E., Junker, U., Hofstetter, H., Wolf, R.M. and Seuwen, K. (2003) Proton-sensing G-protein-coupled receptors. *Nature*, **425**, 93-98.

Lund-Olesen, K. (1970) Oxygen tension in synovial fluids. *Arthritis Rheum*, **13**, 769-776.

Luo, G., Ducky, P., McKee, M.D., Pinero, G.J., Loyer, E., Behringer, R.R. and Karsenty, G. (1997) Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature*, **386**, 78-81.

Ma, T., Miyanishi, K., Suen, A., Epstein, N.J., Tomita, T., Smith, R.L. and Goodman, S.B. (2004) Human interleukin-1-induced murine osteoclastogenesis is dependent on RANKL, but independent of TNF- $\alpha$ . *Cytokine*, **26**, 138-144.

MacDonald, B.R., Takahashi, N., McManus, L.M., Holahan, J., Mundy, G.R. and Roodman, G.D. (1987) Formation of multinucleated cells that respond to osteotropic hormones in long term human bone marrow cultures. *Endocrinology*, **120**, 2326-2333.

Maes, C., Carmeliet, P., Moermans, K., Stockmans, I., Smets, N., Collen, D., Bouillon, R. and Carmeliet, G. (2002) Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Mech Dev*, **111**, 61-73.

Makino, Y., Kanopka, A., Wilson, W.J., Tanaka, H. and Poellinger, L. (2002) Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3 $\alpha$  locus. *J Biol Chem*, **277**, 32405-32408.

Malladi, P., Xu, Y., Chiou, M., Giaccia, A.J. and Longaker, M.T. (2005) The effect of reduced oxygen tension on chondrogenesis and osteogenesis in adipose-derived mesenchymal cells. *Am J Physiol Cell Physiol*, (in press).

Mancini, L., Moradi-Bidhendi, N., Becherini, L., Martinetti, V. and MacIntyre, I. (2000) The biphasic effects of nitric oxide in primary rat osteoblasts are cGMP dependent. *Biochem Biophys Res Commun*, **274**, 477-481.

Massey, H.M. and Flanagan, A.M. (1999) Human osteoclasts derive from CD14<sup>+</sup> positive monocytes. *Br J Haematol*, **106**, 167-170.

Matsuda, N., Morita, N., Matsuda, K. and Watanabe, M. (1998) Proliferation and differentiation of human osteoblastic cells associated with differential activation of

MAP kinases in response to epidermal growth factor, hypoxia, and mechanical stress in vitro. *Biochem Biophys Res Commun*, **249**, 350-354.

Matsuzaki, K., Udagawa, N., Takahashi, N., Yamaguchi, K., Yasuda, H., Shima, N., Morinaga, T., Toyama, Y., Yabe, Y., Higashio, K. and Suda, T. (1998) Osteoclast differentiation factor (ODF) induces osteoclast-like cell formation in human peripheral blood mononuclear cell cultures. *Biochem Biophys Res Commun*, **246**, 199-204.

Mauviel, A. (2005) Transforming growth factor- $\beta$ : a key mediator of fibrosis. *Methods Mol Med*, **117**, 69-80.

Maxwell, P. and Salnikow, K. (2004) HIF-1: an oxygen and metal responsive transcription factor. *Cancer Biol Ther*, **3**, 29-35.

Maxwell, P.H. (2002) Oxygen homeostasis and cancer: insights from a rare disease. *Clin Med*, **2**, 356-362.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R. and Ratcliffe, P.J. (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature*, **399**, 271-275.

Mayer, H., Bertram, H., Lindenmaier, W., Korff, T., Weber, H. and Weich, H. (2005) Vascular endothelial growth factor (VEGF-A) expression in human mesenchymal stem cells: autocrine and paracrine role on osteoblastic and endothelial differentiation. *J Cell Biochem*, **95**, 827-839.

Meghji, S., Morrison, M.S., Henderson, B. and Arnett, T.R. (2001) pH dependence of bone resorption: mouse calvarial osteoclasts are activated by acidosis. *Am J Physiol Endocrinol Metab*, **280**, E112-119.

Mendelsohn, M.E. (2000) Nongenomic, ER-mediated activation of endothelial nitric oxide synthase: how does it work? What does it mean? *Circ Res*, **87**, 956-960.

Mendelsohn, M.E. and Karas, R.H. (1999) The protective effects of estrogen on the cardiovascular system. *N Engl J Med*, **340**, 1801-1811.

Metzen, E., Berchner-Pfannschmidt, U., Stengel, P., Marxsen, J.H., Stolze, I., Klinger, M., Huang, W.Q., Wotzlaw, C., Hellwig-Burgel, T., Jelkmann, W., Acker, H. and Fandrey, J. (2003) Intracellular localisation of human HIF-1  $\alpha$  hydroxylases: implications for oxygen sensing. *J Cell Sci*, **116**, 1319-1326.

Min, J.K., Kim, Y.M., Kim, Y.M., Kim, E.C., Gho, Y.S., Kang, I.J., Lee, S.Y., Kong, Y.Y. and Kwon, Y.G. (2003) Vascular endothelial growth factor up-regulates expression of receptor activator of NF- $\kappa$ B (RANK) in endothelial cells: Concomitant increase of angiogenic responses to RANK ligand. *J Biol Chem*, **278**, 39548-39557.

Miyazaki, T., Sanjay, A., Neff, L., Tanaka, S., Horne, W.C. and Baron, R. (2004) Src kinase activity is essential for osteoclast function. *J Biol Chem*, **279**, 17660-17666.

Moncada, S., Palmer, R.M. and Higgs, E.A. (1988) The discovery of nitric oxide as the endogenous nitrovasodilator. *Hypertension*, **12**, 365-372.

Mulari, M., Vaaraniemi, J. and Vaananen, H.K. (2003) Intracellular membrane trafficking in bone resorbing osteoclasts. *Microsc Res Tech*, **61**, 496-503.

Mundy, G., Garrett, R., Harris, S., Chan, J., Chen, D., Rossini, G., Boyce, B., Zhao, M. and Gutierrez, G. (1999) Stimulation of bone formation *in vitro* and in rodents by statins. *Science*, **286**, 1946-1949.

Murdoch, C., Muthana, M. and Lewis, C.E. (2005) Hypoxia regulates macrophage functions in inflammation. *J Immunol*, **175**, 6257-6263.

Murshed, M., Schinke, T., McKee, M.D. and Karsenty, G. (2004) Extracellular matrix mineralization is regulated locally; different roles of two gla-containing proteins. *J Cell Biol*, **165**, 625-630.

Nagata, N., Kitaura, H., Yoshida, N. and Nakayama, K. (2003) Inhibition of RANKL-induced osteoclast formation in mouse bone marrow cells by IL-12:

involvement of IFN-gamma possibly induced from non-T cell population. *Bone*, **33**, 721-732.

Nakagawa, M., Kaneda, T., Arakawa, T., Morita, S., Sato, T., Yomada, T., Hanada, K., Kumegawa, M. and Hakeda, Y. (2000) Vascular endothelial growth factor (VEGF) directly enhances osteoclastic bone resorption and survival of mature osteoclasts. *FEBS Lett*, **473**, 161-164.

Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J.M., Behringer, R.R. and de Crombrughe, B. (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell*, **108**, 17-29.

Nakashima, T., Kobayashi, Y., Yamasaki, S., Kawakami, A., Eguchi, K., Sasaki, H. and Sakai, H. (2000) Protein expression and functional difference of membrane-bound and soluble receptor activator of NF- $\kappa$ B ligand: modulation of the expression by osteotropic factors and cytokines. *Biochem Biophys Res Commun*, **275**, 768-775.

Niida, S., Kaku, M., Amano, H., Yoshida, H., Kataoka, H., Nishikawa, S., Tanne, K., Maeda, N., Nishikawa, S. and Kodama, H. (1999) Vascular endothelial growth factor can substitute for macrophage colony-stimulating factor in the support of osteoclastic bone resorption. *J Exp Med*, **190**, 293-298.

Noble, B.S. and Reeve, J. (2000) Osteocyte function, osteocyte death and bone fracture resistance. *Mol Cell Endocrinol*, **159**, 7-13.

Norman, J.T., Clark, I.M. and Garcia, P.L. (2000) Hypoxia promotes fibrogenesis in human renal fibroblasts. *Kidney Int*, **58**, 2351.

O' Gradaigh, D., Ireland, D., Bord, S. and Compston, J.E. (2004) Joint erosion in rheumatoid arthritis: interactions between tumour necrosis factor  $\alpha$ , interleukin 1, and receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) regulate osteoclasts. *Ann Rheum Dis*, **63**, 354-359.

Oberringer, M., Jennewein, M., Motsch, S.E., Pohlemann, T. and Seekamp, A. (2005) Different cell cycle responses of wound healing protagonists to transient in vitro hypoxia. *Histochem Cell Biol*, **123**, 595-603.

Ontiveros, C., Irwin, R., Wiseman, R.W. and McCabe, L.R. (2004) Hypoxia suppresses runx2 independent of modeled microgravity. *J Cell Physiol*, **200**, 169-176.

Ornitz, D.M. (2005) FGF signaling in the developing endochondral skeleton. *Cytokine Growth Factor Rev*, **16**, 205-213.

Otto, F., Thornell, A.P., Crompton, T., Denzel, A., Gilmour, K.C., Rosewell, I.R., Stamp, G.W., Beddington, R.S., Mundlos, S., Olsen, B.R., Selby, P.B. and Owen, M.J. (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell*, **89**, 765-771.

Oxlund, H., Barckman, M., Ortoft, G. and Andreassen, T.T. (1995) Reduced concentrations of collagen cross-links are associated with reduced strength of bone. *Bone*, **17**, 365S-371S.

Oxlund, H., Mosekilde, L. and Ortoft, G. (1996) Reduced concentration of collagen reducible cross links in human trabecular bone with respect to age and osteoporosis. *Bone*, **19**, 479-484.

Park, J.H., Park, B.H., Kim, H.K., Park, T.S. and Baek, H.S. (2002) Hypoxia decreases Runx2/Cbfa1 expression in human osteoblast-like cells. *Mol Cell Endocrinol*, **192**, 197-203.

Peers, C. and Kemp, P.J. (2004) Ion channel regulation by chronic hypoxia in models of acute oxygen sensing. *Cell Calcium*, **36**, 341-348.

Peyssonaux, C. and Johnson, R.S. (2004) An unexpected role for hypoxic response: oxygenation and inflammation. *Cell Cycle*, **3**, 168-171.

Pfau, J.C., Schneider, J.C., Archer, A.J., Sentissi, J., Leyva, F.J. and Cramton, J. (2004) Environmental oxygen tension affects phenotype in cultured bone marrow-derived macrophages. *Am J Physiol Lung Cell Mol Physiol*, **286**, L354-362.

Pilbeam, C.C., Raisz, L.G., Voznesensky, O., Alander, C.B., Delman, B.N. and Kawaguchi, H. (1995) Autoregulation of inducible prostaglandin G/H synthase in osteoblastic cells by prostaglandins. *J Bone Miner Res*, **10**, 406-414.

Prockop, D.J. (1990) Mutations that alter the primary structure of type I collagen. The perils of a system for generating large structures by the principle of nucleated growth. *J Biol Chem*, **265**, 15349-15352.

Provot, S. and Schipani, E. (2005) Molecular mechanisms of endochondral bone development. *Biochem Biophys Res Commun*, **328**, 658-665.

Purroy, J. and Spurr, N.K. (2002) Molecular genetics of calcium sensing in bone cells. *Hum Mol Genet*, **11**, 2377-2384.

Qu, Q., Perala-Heape, M., Kapanen, A., Dahllund, J., Salo, J., Vaananen, H.K. and Harkonen, P. (1998) Estrogen enhances differentiation of osteoblasts in mouse bone marrow culture. *Bone*, **22**, 201-209.

Quinn, J.M., Itoh, K., Udagawa, N., Hausler, K., Yasuda, H., Shima, N., Mizuno, A., Higashio, K., Takahashi, N., Suda, T., Martin, T.J. and Gillespie, M.T. (2001) Transforming growth factor  $\beta$  affects osteoclast differentiation via direct and indirect actions. *J Bone Miner Res*, **16**, 1787-1794.

Raisz, L.G. (1999) Prostaglandins and bone: physiology and pathophysiology. *Osteoarthritis Cartilage*, **7**, 419-421.

Rajpurohit, R., Koch, C.J., Tao, Z., Teixeira, C.M. and Shapiro, I.M. (1996) Adaptation of chondrocytes to low oxygen tension: relationship between hypoxia and cellular metabolism. *J Cell Physiol*, **168**, 424-432.

Ramaley, P.B. and Rosenbloom, J. (1971) Inhibition of proline and lysine hydroxylation prevents normal extrusion of collagen by 3T6 fibroblasts in culture. *FEBS Lett*, **15**, 59-64.

Reid, I.R. (1997) Glucocorticoid osteoporosis--mechanisms and management. *Eur J Endocrinol*, **137**, 209-217.

Reseland, J.E., Syversen, U., Bakke, I., Qvigstad, G., Eide, L.G., Hjertner, O., Gordeladze, J.O. and Drevon, C.A. (2001) Leptin is expressed in and secreted from

primary cultures of human osteoblasts and promotes bone mineralization. *J Bone Miner Res*, **16**, 1426-1433.

Reszka, A.A. and Rodan, G.A. (2003) Bisphosphonate mechanism of action. *Curr Rheumatol Rep*, **5**, 65-74.

Roberts-Harry, D. and Sandy, J. (2004) Orthodontics. Part 11: orthodontic tooth movement. *Br Dent J*, **196**, 391-394.

Rodriguez-Tarduchy, G., Collins, M.K., Garcia, I. and Lopez-Rivas, A. (1992) Insulin-like growth factor-I inhibits apoptosis in IL-3-dependent hemopoietic cells. *J Immunol*, **149**, 535-540.

Ryan, H.E., Lo, J. and Johnson, R.S. (1998) HIF-1  $\alpha$  is required for solid tumor formation and embryonic vascularization. *Embo J*, **17**, 3005-3015.

Saed, G.M. and Diamond, M.P. (2002) Hypoxia-induced irreversible up-regulation of type I collagen and transforming growth factor- $\beta$ 1 in human peritoneal fibroblasts. *Fertil Steril*, **78**, 144.

Saed, G.M., Munkarah, A.R., Abu-Soud, H.M. and Diamond, M.P. (2005) Hypoxia upregulates cyclooxygenase-2 and prostaglandin E<sub>2</sub> levels in human peritoneal fibroblasts. *Fertil Steril*, **83 Suppl 1**, 1216-1219.

Saftig, P., Hunziker, E., Wehmeyer, O., Jones, S., Boyde, A., Rommerskirch, W., Moritz, J.D., Schu, P. and von Figura, K. (1998) Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. *Proc Natl Acad Sci U S A*, **95**, 13453-13458.

Sahai, A., Patel, M.S., Zavosh, A.S. and Tannen, R.L. (1994) Chronic hypoxia impairs the differentiation of 3T3-L1 fibroblast in culture: role of sustained protein kinase C activation. *J Cell Physiol*, **160**, 107-112.

Salim, A., Nacamuli, R.P., Morgan, E.F., Giaccia, A.J. and Longaker, M.T. (2004) Transient changes in oxygen tension inhibit osteogenic differentiation and Runx2 expression in osteoblasts. *J Biol Chem*, **279**, 40007-40016.



Sanjay, A., Houghton, A., Neff, L., DiDomenico, E., Bardelay, C., Antoine, E., Levy, J., Gailit, J., Bowtell, D., Horne, W.C. and Baron, R. (2001) Cbl associates with Pyk2 and Src to regulate Src kinase activity,  $\alpha_v\beta_3$  integrin-mediated signaling, cell adhesion, and osteoclast motility. *J Cell Biol*, **152**, 181-195.

Santhanagopal, A. and Dixon, S.J. (1999) Insulin-like growth factor I rapidly enhances acid efflux from osteoblastic cells. *Am J Physiol*, **277**, E423-432.

Sarma, U., Edwards, M., Motoyoshi, K. and Flanagan, A.M. (1998) Inhibition of bone resorption by 17 $\beta$ -estradiol in human bone marrow cultures. *J Cell Physiol*, **175**, 99-108.

Scheid, A., Wenger, R.H., Schaffer, L., Camenisch, I., Distler, O., Ferenc, A., Cristina, H., Ryan, H.E., Johnson, R.S., Wagner, K.F., Stauffer, U.G., Bauer, C., Gassmann, M. and Meuli, M. (2002) Physiologically low oxygen concentrations in fetal skin regulate hypoxia-inducible factor 1 and transforming growth factor- $\beta_3$ . *Faseb J*, **16**, 411-413.

Schiff, M.H. (2000) Role of interleukin 1 and interleukin 1 receptor antagonist in the mediation of rheumatoid arthritis. *Ann Rheum Dis*, **59 Suppl 1**, i103-108.

Schipani, E., Ryan, H.E., Didrickson, S., Kobayashi, T., Knight, M. and Johnson, R.S. (2001) Hypoxia in cartilage: HIF-1 $\alpha$  is essential for chondrocyte growth arrest and survival. *Genes Dev*, **15**, 2865-2876.

Schreck, R., Rieber, P. and Baeuerle, P.A. (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF- $\kappa$ B transcription factor and HIV-1. *Embo J*, **10**, 2247-2258.

Scortegagna, M., Ding, K., Oktay, Y., Gaur, A., Thurmond, F., Yan, L.J., Marck, B.T., Matsumoto, A.M., Shelton, J.M., Richardson, J.A., Bennett, M.J. and Garcia, J.A. (2003) Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1<sup>-/-</sup> mice. *Nat Genet*, **35**, 331-340.

Scortegagna, M., Morris, M.A., Oktay, Y., Bennett, M. and Garcia, J.A. (2003) The HIF family member EPAS1/HIF-2 $\alpha$  is required for normal hematopoiesis in mice. *Blood*, **102**, 1634-1640.

Semenza, G.L. (1999) Regulation of mammalian O<sub>2</sub> homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol*, **15**, 551-578.

Semenza, G.L. (2000) Oxygen-regulated transcription factors and their role in pulmonary disease. *Respir Res*, **1**, 159-162.

Semenza, G.L. (2001) Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. *Trends Mol Med*, **7**, 345-350.

Serre, C.M., Farlay, D., Delmas, P.D. and Chenu, C. (1999) Evidence for a dense and intimate innervation of the bone tissue, including glutamate-containing fibers. *Bone*, **25**, 623.

Seta, K.A., Yuan, Y., Spicer, Z., Lu, G., Bedard, J., Ferguson, T.K., Pathrose, P., Cole-Strauss, A., Kaufhold, A. and Millhorn, D.E. (2004) The role of calcium in hypoxia-induced signal transduction and gene expression. *Cell Calcium*, **36**, 331-340.

Shattil, S.J. (2005) Integrins and Src: dynamic duo of adhesion signaling. *Trends Cell Biol*, **15**, 399-403.

Shen, J., Bronson, R.T., Chen, D.F., Xia, W., Selkoe, D.J. and Tonegawa, S. (1997) Skeletal and CNS defects in Presenilin-1-deficient mice. *Cell*, **89**, 629-639.

Shimamura, T., Amizuka, N., Li, M., Freitas, P.H., White, J.H., Henderson, J.E., Shingaki, S., Nakajima, T. and Ozawa, H. (2005) Histological observations on the microenvironment of osteolytic bone metastasis by breast carcinoma cell line. *Biomed Res*, **26**, 159-172.

Shinoda, K., Sugiyama, E., Taki, H., Harada, S., Mino, T., Maruyama, M. and Kobayashi, M. (2003) Resting T cells negatively regulate osteoclast generation from peripheral blood monocytes. *Bone*, **33**, 711-720.

Soriano, P., Montgomery, C., Geske, R. and Bradley, A. (1991) Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell*, **64**, 693-702.

Soyka, L.A., Misra, M., Frenchman, A., Miller, K.K., Grinspoon, S., Schoenfeld, D.A. and Klibanski, A. (2002) Abnormal bone mineral accrual in adolescent girls with anorexia nervosa. *J Clin Endocrinol Metab*, **87**, 4177-4185.

Spencer, G.J., Utting, J.C., Etheridge, S.L., Arnett, T.R. and Genever, P.G. (2005) Wnt signalling in osteoblasts regulates expression of receptor activator of NF $\kappa$ B ligand (RANKL) and inhibits osteoclastogenesis in vitro. *J Cell Sci*, (In press).

Stains, J.P. and Civitelli, R. (2005) Cell-to-cell interactions in bone. *Biochem Biophys Res Commun*, **328**, 721-727.

Stanley, E.R., Berg, K.L., Einstein, D.B., Lee, P.S., Pixley, F.J., Wang, Y. and Yeung, Y.G. (1997) Biology and action of colony--stimulating factor-1. *Mol Reprod Dev*, **46**, 4-10.

Steinbrech, D.S., Longaker, M.T., Mehrara, B.J., Saadeh, P.B., Chin, G.S., Gerrets, R.P., Chau, D.C., Rowe, N.M. and Gittes, G.K. (1999) Fibroblast response to hypoxia: the relationship between angiogenesis and matrix regulation. *J Surg Res*, **84**, 127-133.

Steinbrech, D.S., Mehrara, B.J., Saadeh, P.B., Chin, G., Dudziak, M.E., Gerrets, R.P., Gittes, G.K. and Longaker, M.T. (1999) Hypoxia regulates VEGF expression and cellular proliferation by osteoblasts in vitro. *Plast Reconstr Surg*, **104**, 738-747.

Steinbrech, D.S., Mehrara, B.J., Saadeh, P.B., Greenwald, J.A., Spector, J.A., Gittes, G.K. and Longaker, M.T. (2000) Hypoxia increases insulinlike growth factor gene expression in rat osteoblasts. *Ann Plast Surg*, **44**, 529-535.

Steinbrech, D.S., Mehrara, B.J., Saadeh, P.B., Greenwald, J.A., Spector, J.A., Gittes, G.K. and Longaker, M.T. (2000) VEGF expression in an osteoblast-like cell line is regulated by a hypoxia response mechanism. *Am J Physiol Cell Physiol*, **278**, C853-C860.

Sterling, H., Saginario, C. and Vignery, A. (1998) CD44 occupancy prevents macrophage multinucleation. *J Cell Biol*, **143**, 837-847.

Stewart, A.J., Houston, B. and Farquharson, C. (2006) Elevated expression of hypoxia inducible factor-2 $\alpha$  in terminally differentiating growth plate chondrocytes. *J Cell Physiol*, **206**, 435-440.

Street, J., Bao, M., deGuzman, L., Bunting, S., Peale, F.V., Jr., Ferrara, N., Steinmetz, H., Hoeffel, J., Cleland, J.L., Daugherty, A., van Bruggen, N., Redmond, H.P., Carano, R.A. and Filvaroff, E.H. (2002) Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc Natl Acad Sci U S A*, **99**, 9656-9661.

Swartz, H.M. (2002) Measuring real levels of oxygen in vivo: opportunities and challenges. *Biochem Soc Trans*, **30**, 248-252.

Syed, F. and Khosla, S. (2005) Mechanisms of sex steroid effects on bone. *Biochem Biophys Res Commun*, **328**, 688-696.

Takahashi, N., Akatsu, T., Udagawa, N., Sasaki, T., Yamaguchi, A., Moseley, J.M., Martin, T.J. and Suda, T. (1988) Osteoblastic cells are involved in osteoclast formation. *Endocrinology*, **123**, 2600-2602.

Takayanagi, H. (2005) Mechanistic insight into osteoclast differentiation in osteoimmunology. *J Mol Med*, **83**, 170-179.

Takayanagi, H., Kim, S., Koga, T., Nishina, H., Isshiki, M., Yoshida, H., Saiura, A., Isobe, M., Yokochi, T., Inoue, J., Wagner, E.F., Mak, T.W., Kodama, T. and Taniguchi, T. (2002) Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell*, **3**, 889-901.

Takayanagi, H., Kim, S. and Taniguchi, T. (2002) Signaling crosstalk between RANKL and interferons in osteoclast differentiation. *Arthritis Res*, **4 Suppl 3**, S227-232.

Take, I., Kobayashi, Y., Yamamoto, Y., Tsuboi, H., Ochi, T., Uematsu, S., Okafuji, N., Kurihara, S., Udagawa, N. and Takahashi, N. (2005) Prostaglandin E<sub>2</sub> strongly inhibits human osteoclast formation. *Endocrinology*, **146**, 5204-5214.

Talks, K.L., Turley, H., Gatter, K.C., Maxwell, P.H., Pugh, C.W., Ratcliffe, P.J. and Harris, A.L. (2000) The expression and distribution of the hypoxia-inducible factors HIF-1 $\alpha$  and HIF-2 $\alpha$  in normal human tissues, cancers, and tumor-associated macrophages. *Am J Pathol*, **157**, 411-421.

Tanabe, N., Maeno, M., Suzuki, N., Fujisaki, K., Tanaka, H., Ogiso, B. and Ito, K. (2005) IL-1 $\alpha$  stimulates the formation of osteoclast-like cells by increasing M-CSF and PGE<sub>2</sub> production and decreasing OPG production by osteoblasts. *Life Sci*, **77**, 615-626.

Tanaka, S., Nakamura, I., Inoue, J., Oda, H. and Nakamura, K. (2003) Signal transduction pathways regulating osteoclast differentiation and function. *J Bone Miner Metab*, **21**, 123-133.

Tandara, A.A. and Mustoe, T.A. (2004) Oxygen in wound healing--more than a nutrient. *World J Surg*, **28**, 294-300.

Tanimoto, K., Makino, Y., Pereira, T. and Poellinger, L. (2000) Mechanism of regulation of the hypoxia-inducible factor-1 $\alpha$  by the von Hippel-Lindau tumor suppressor protein. *Embo J*, **19**, 4298-4309.

Tavassoli, M. and Yoffey, J.M. (1983) *Bone Marrow: Structure and Function*. Alan R. Liss, Inc., New York.

Taylor, P.C. and Sivakumar, B. (2005) Hypoxia and angiogenesis in rheumatoid arthritis. *Curr Opin Rheumatol*, **17**, 293-298.

Teitelbaum, S.L. (2000) Bone resorption by osteoclasts. *Science*, **289**, 1504-1508.

Therade-Matharan, S., Laemmel, E., Duranteau, J. and Vicaut, E. (2004) Reoxygenation after hypoxia and glucose depletion causes reactive oxygen species

production by mitochondria in HUVEC. *Am J Physiol Regul Integr Comp Physiol*, **287**, R1037-1043.

Thomson, B.M., Mundy, G.R. and Chambers, T.J. (1987) Tumor necrosis factors  $\alpha$  and  $\beta$  induce osteoblastic cells to stimulate osteoclastic bone resorption. *J Immunol*, **138**, 775-779.

Tian, H., Hammer, R.E., Matsumoto, A.M., Russell, D.W. and McKnight, S.L. (1998) The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. *Genes Dev*, **12**, 3320-3324.

Tombran-Tink, J. and Barnstable, C.J. (2004) Osteoblasts and osteoclasts express PEDF, VEGF-A isoforms, and VEGF receptors: possible mediators of angiogenesis and matrix remodeling in the bone. *Biochem Biophys Res Commun*, **316**, 573-579.

Tondravi, M.M., McKercher, S.R., Anderson, K., Erdmann, J.M., Quiroz, M., Maki, R. and Teitelbaum, S.L. (1997) Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. *Nature*, **386**, 81-84.

Treins, C., Giorgetti-Peraldi, S., Murdaca, J., Monthouel-Kartmann, M.N. and Van Obberghen, E. (2005) Regulation of hypoxia-inducible factor (HIF)-1 activity and expression of HIF hydroxylases in response to insulin-like growth factor I. *Mol Endocrinol*, **19**, 1304-1317.

Troen, B.R. (2003) Molecular mechanisms underlying osteoclast formation and activation. *Exp Gerontol*, **38**, 605-614.

Uchida, T., Rossignol, F., Matthay, M.A., Mounier, R., Couette, S., Clottes, E. and Clerici, C. (2004) Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1 $\alpha$  and HIF-2 $\alpha$  expression in lung epithelial cells: implication of natural antisense HIF-1 $\alpha$ . *J Biol Chem*, **279**, 14871-14878.

Udagawa, N. (2003) The mechanism of osteoclast differentiation from macrophages: possible roles of T lymphocytes in osteoclastogenesis. *J Bone Miner Metab*, **21**, 337-343.

Udagawa, N., Takahashi, N., Jimi, E., Matsuzaki, K., Tsurukai, T., Itoh, K., Nakagawa, N., Yasuda, H., Goto, M., Tsuda, E., Higashio, K., Gillespie, M.T., Martin, T.J. and Suda, T. (1999) Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor: receptor activator of NF- $\kappa$ B ligand. *Bone*, **25**, 517-523.

Utting, J., Brandao-Burch, A. and Arnett, T. (2004) Severe hypoxia increases osteoclast formation from human peripheral blood: amplification of resorption by acidosis. *Calcif Tissue Int*, **74**, S63 (abstract).

Vaaranieniemi, J., Halleen, J.M., Kaarlonen, K., Ylipahkala, H., Alatalo, S.L., Andersson, G., Kaija, H., Vihko, P. and Vaananen, H.K. (2004) Intracellular machinery for matrix degradation in bone-resorbing osteoclasts. *J Bone Miner Res*, **19**, 1432-1440.

Vaupel, P., Kallinowski, F. and Okunieff, P. (1989) Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res*, **49**, 6449-6465.

Volterrani, M., Rosano, G., Coats, A., Beale, C. and Collins, P. (1995) Estrogen acutely increases peripheral blood flow in postmenopausal women. *Am J Med*, **99**, 119-122.

Vu, T.H., Shipley, J.M., Bergers, G., Berger, J.E., Helms, J.A., Hanahan, D., Shapiro, S.D., Senior, R.M. and Werb, Z. (1998) MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell*, **93**, 411-422.

Walmsley, S.R., Print, C., Farahi, N., Peyssonnaud, C., Johnson, R.S., Cramer, T., Sobolewski, A., Condliffe, A.M., Cowburn, A.S., Johnson, N. and Chilvers, E.R. (2005) Hypoxia-induced neutrophil survival is mediated by HIF-1 $\alpha$ -dependent NF- $\kappa$ B activity. *J Exp Med*, **201**, 105-115.

Wan, M. and Cao, X. (2005) BMP signaling in skeletal development. *Biochem Biophys Res Commun*, **328**, 651-657.

Wang, L., Ciani, C., Doty, S.B. and Fritton, S.P. (2004) Delineating bone's interstitial fluid pathway in vivo. *Bone*, **34**, 499-509.

Wang, L., Cowin, S.C., Weinbaum, S. and Fritton, S.P. (2000) Modeling tracer transport in an osteon under cyclic loading. *Ann Biomed Eng*, **28**, 1200-1209.

Warren, S.M., Steinbrech, D.S., Mehrara, B.J., Saadeh, P.B., Greenwald, J.A., Spector, J.A., Bouletreau, P.J. and Longaker, M.T. (2001) Hypoxia regulates osteoblast gene expression. *J Surg Res*, **99**, 147-155.

Watanuki, M., Sakai, A., Sakata, T., Tsurukami, H., Miwa, M., Uchida, Y., Watanabe, K., Ikeda, K. and Nakamura, T. (2002) Role of inducible nitric oxide synthase in skeletal adaptation to acute increases in mechanical loading. *J Bone Miner Res*, **17**, 1015-1025.

Waypa, G.B., Chandel, N.S. and Schumacker, P.T. (2001) Model for hypoxic pulmonary vasoconstriction involving mitochondrial oxygen sensing. *Circ Res*, **88**, 1259-1266.

Wei, S., Wang, M.W., Teitelbaum, S.L. and Ross, F.P. (2002) Interleukin-4 reversibly inhibits osteoclastogenesis via inhibition of NF- $\kappa$ B and mitogen-activated protein kinase signaling. *J Biol Chem*, **277**, 6622-6630.

Westendorf, J.J., Kahler, R.A. and Schroeder, T.M. (2004) Wnt signaling in osteoblasts and bone diseases. *Gene*, **341**, 19-39.

Wheeler, P. and Batt, M.E. (2005) Do non-steroidal anti-inflammatory drugs adversely affect stress fracture healing? A short review. *Br J Sports Med*, **39**, 65-69.

Whitney, C., Warburton, D.E., Frohlich, J., Chan, S.Y., McKay, H. and Khan, K. (2004) Are cardiovascular disease and osteoporosis directly linked? *Sports Med*, **34**, 779-807.



Williams, J.P., Blair, H.C., McDonald, J.M., McKenna, M.A., Jordan, S.E., Williford, J. and Hardy, R.W. (1997) Regulation of osteoclastic bone resorption by glucose. *Biochem Biophys Res Commun*, **235**, 646-651.

Williamson, P.R. and Kagan, H.M. (1986) Reaction pathway of bovine aortic lysyl oxidase. *J Biol Chem*, **261**, 9477-9482.

Winet, H., Hsieh, A. and Bao, J.Y. (1998) Approaches to study of ischemia in bone. *J Biomed Mater Res*, **43**, 410-421.

Wong, G.L. and Cohn, D.V. (1975) Target cells in bone for parathormone and calcitonin are different: enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces. *Proc Natl Acad Sci U S A*, **72**, 3167-3171.

Yao, G.Q., Itokawa, T., Paliwal, I. and Insogna, K. (2005) CSF-1 induces fos gene transcription and activates the transcription factor Elk-1 in mature osteoclasts. *Calcif Tissue Int*, **76**, 371-378.

Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomoyasu, A., Yano, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., Higashio, K., Udagawa, N., Takahashi, N. and Suda, T. (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A*, **95**, 3597-3602.

Yun, Z., Maecker, H.L., Johnson, R.S. and Giaccia, A.J. (2002) Inhibition of PPAR $\gamma$ 2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for regulation of adipogenesis by hypoxia. *Dev Cell*, **2**, 331-341.

Zaman, G., Pitsillides, A.A., Rawlinson, S.C., Suswillo, R.F., Mosley, J.R., Cheng, M.Z., Platts, L.A., Hukkanen, M., Polak, J.M. and Lanyon, L.E. (1999) Mechanical strain stimulates nitric oxide production by rapid activation of endothelial nitric oxide synthase in osteocytes. *J Bone Miner Res*, **14**, 1123-1131.

Zelzer, E., McLean, W., Ng, Y.S., Fukai, N., Reginato, A.M., Lovejoy, S., D'Amore, P.A. and Olsen, B.R. (2002) Skeletal defects in VEGF(120/120) mice reveal multiple roles for VEGF in skeletogenesis. *Development*, **129**, 1893-1904.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., Harris, S., Wiedemann, L.M., Mishina, Y. and Li, L. (2003) Identification of the haematopoietic stem cell niche and control of the niche size. *Nature*, **425**, 836-841.

Zhang, M., Xuan, S., Bouxsein, M.L., von Stechow, D., Akeno, N., Faugere, M.C., Malluche, H., Zhao, G., Rosen, C.J., Efstratiadis, A. and Clemens, T.L. (2002) Osteoblast-specific knockout of the insulin-like growth factor (IGF) receptor gene reveals an essential role of IGF signaling in bone matrix mineralization. *J Biol Chem*, **277**, 44005-44012.

Zhou, S., Cui, Z. and Urban, J.P. (2004) Factors influencing the oxygen concentration gradient from the synovial surface of articular cartilage to the cartilage-bone interface: a modeling study. *Arthritis Rheum*, **50**, 3915-3924.

# Appendix I

## *Abbreviations*

ADP	Adenosine diphosphate
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
API	Activator protein 1
ARNT	Aryl hydrocarbon nuclear translocator
ASICS	Acid sensitive ion channel
ATP	Adenosine triphosphate
BAPN	Beta amino proprionitrile
BMD	Bone mineral density
BMP	Bone morphogenic protein
BP	Bisphosphonate
BSA	Bovine serum albumin
BSP	Bone sialoprotein
CatK	Cathepsin K
cDNA	Clone deoxyribonucleic acid
cGMP	cyclic guanine monophosphate
COPD	Chronic obstructive pulmonary disease
COX2	Cyclooxygenase 2
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified essential medium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ERK	Extracellular signal regulated kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FIH	Factor inhibiting HIF
FITC	Fluorescence isothiocyanate
FOXY	Fibre optic oxygen
GC	Glucocorticoid
GM-CSF	Granulocyte macrophage - colony stimulating factor
HIF	Hypoxia inducible factor

HOX	Homeobox
HRP	Horseradish peroxidase
I $\kappa$ B	Inhibitor of $\kappa$ B
IFN	Interferon
IGF	Insulin-like growth factor
Ihh	Indian hedgehog
IKK	Inhibitor of $\kappa$ B kinase
IL	Interleukin
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LEF	Lymphocyte enhancement factor
LOX	Lysyl oxidase
LPS	Lipopolysaccharide
LRP5	LDL receptor related protein 5
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage - colony stimulating factor
MEM	Minimal essential medium
Mitf	Microphthalmia-associated transcription factor
MMP	Matrix metalloproteinase
mRNA	messenger ribonucleic acid
NFATc1	cytoplasmic nuclear factor of activated T cells
NF $\kappa$ B	Nuclear factor $\kappa$ B
NHDF	normal human dermal fibroblast
nNOS	neuronal nitric oxide synthase
NO	Nitric oxide
OA	Osteoarthritis
OB	Osteoblast
OC	Osteoclast
OCN	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
Osx	Osterix
PBMNC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDI	Protein disulphide isomerase
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>
PHD	Prolyl hydroxylase
PLOD	Procollagen-lysine, 2-oxoglutarate, 5-dioxygenase
PMSF	Phenyl methyl sulfonyl fluoride
P4OH	Prolyl 4-hydroxylase
PTH/PTHrP	Parathyroid hormone / parathyroid hormone related peptide

PVDF	Polyvinylidene difluoride
pVHL	Von Hippel Lindau tumour suppressor protein
RA	Rheumatoid arthritis
RANK	Receptor activator of NFκB
RANKL	Receptor activator of NFκB ligand
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SEM	Standard error measurement
STAT	Signal transducers and activators of transcription
TCA	Trichloroacetic acid
TCF	T cell factor
TGF	Transforming growth factor
TNFα	Tumour necrosis factor α
TRACP	Tartrate resistant acid phosphatase
TRAF	TNF receptor associated factor
TRAIL	TNF receptor apoptosis inducing ligand
TUNEL	TdT-mediated dUTP-dioxigenin nick end labelling
UTP	Uridine triphosphate
VEGF	Vascular endothelial growth factor
Vitamin D	1, 25- hydroxy vitamin D <sub>3</sub>
VNR	Vitronectin receptor
VSMC	Vascular smooth muscle cell

# Appendix II

## *Publications*

### *Papers*

Arnett, T.R., Gibbons, D.C., Utting, J.C., Orriss, I.R., Hoebertz, A., Rosendaal, M. and Meghji, S. (2003) Hypoxia is a major stimulator of osteoclast formation and bone resorption. *J Cell Physiol*, **196**(1), 2-8.

Brandao-Burch, A., Utting, J.C., Orriss, I.R. and Arnett, T.R. (2005) Acidosis inhibits bone formation by osteoblasts in vitro by preventing mineralization. *Calcif Tissue Int*, **77**, 167-174.

Spencer, G.J., Utting, J.C., Etheridge, S.L., Arnett, T.R. and Genever, P.G. (2006) Wnt signalling in osteoblasts regulates expression of receptor activator of NFκB ligand (RANKL) and inhibits osteoclastogenesis in vitro. *J Cell Sci* (*in press*).

Utting, J.C., Robins, S.P., Brandao-Burch, A., Orriss, I.R., Behar, J. and Arnett, T.R. (2006) Hypoxia inhibits the growth, differentiation and bone forming capacity of rat osteoblasts. *Exp Cell Res* (*in press*).

Utting, J.C., Flanagan, A.M., Massey, H.M., Brandao-Burch, A., Orriss, I.R. and Arnett, T.R. (2006) Hypoxia stimulates osteoclast formation from human peripheral blood. *Blood* (*manuscript in preparation*).

### *Abstracts*

Utting, J.C., Lee, A., Brandao-Burch, A., Orriss, I.R. and Arnett, T.R. (2005) Hypoxia inhibits cartilage formation in micromass cultures. *J Bone Miner Res*, **20**(7), 1304.

Utting, J.C., Brandao-Burch, A., Orriss, I.R., Whatling, P.J. and Arnett, T.R. (2005) The effect of temperature on bone cell function. *Bone*, **36**, S268.

Brandao-Burch, A., Utting, J.C., Orriss, I.R. and Arnett, T.R. (2004) Osteoblast function is inhibited cooperatively by acidosis and hypoxia. *J Bone Miner Res*, **19**, 1031.

Utting, J.C., Brandao-Burch, A. and Arnett, T.R. (2004) Severe hypoxia increases osteoclast formation from human peripheral blood: amplification of resorption by acidosis. *Calcif Tissue Int*, **74 (Supl 1)**, S63.

Brandao-Burch, A., Orriss, I.R., Utting, J.C. and Arnett, T.R. (2004) Cooperative inhibition of osteoblast function by acidosis and hypoxia. *Calcif Tissue Int*, **74 (Supl 1)**, S55.

Utting, J.C., Brandao-Burch, A. and Arnett, T.R. (2004) Effects of hypoxia and acidosis on the formation and activation of human osteoclasts: evidence for a key role of the vasculature in bone remodelling. *Bone*, **34(Sup 1)**, S28-9.

Arnett, T.R., Massey, H., Utting, J.C., Orriss, I.R. and Flanagan, A.M. (2003) Hypoxia is a major stimulator of osteoclast formation from human peripheral blood. *Calcif Tissue Int*, **72(4)**, 345-6.

Orriss, I.R., Brandao-Burch, A., Utting, J.C. and Arnett, T.R. (2003) Hypoxia and acidosis disrupt bone nodule formation by rat osteoblasts. *Calcif Tissue Int*, **72(4)**, 361.

Utting, J.C., Orriss, I.R., Brandao-Burch, A. and Arnett, T.R. (2003) Severe hypoxia stimulates formation but not activity of rodent osteoclasts. *Calcif Tissue Int*, **72(4)**, 370.

































